# PENTOSE PHOSPHATE PATHWAY REACTIONS

# IN NORMAL, REGENERATING, FOETAL

# AND NEOPLASTIC HEPATIC TISSUE

A Thesis submitted for the Degree of

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by

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## STATEMENT

This thesis reports the results of research which was carried out in the Department of Biochemistry, Faculty of Science, at the Australian National University, Canberra, under the supervision of Professor J.F. Williams. The material incorporated in this thesis is my own work and has not been submitted towards a degree in any other university.

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## ABSTRACT

There are two known reaction sequences for the non-oxidative segment of the pentose phosphate pathway in tissues. The first of these was proposed by Horecker *et al*. (1954) and is considered to be the only variant of the pathway operating in adipose tissue. This reaction sequence is referred to as the F-type (fat cell) pathway or cycle. The second reaction sequence was originally proposed by Williams and Clark (1971) and confirmed to operate in liver *in vitro* by Williams *et al*. (1978 a,b). This reaction sequence is referred to as the L-type (liver) pathway or cycle. It has not been possible to reach an agreement regarding the quantitative role of the pentose phosphate pathway in liver due to the variability of a number of estimates. It is considered that this variability is due in large part to the use of theoretical expressions and experimental approaches for estimating the participation of the pathway based on the F-type reaction sequence and which are not valid for the L-type pathway or cycle. The investigations reported in this thesis are concerned with the estimation of the quantitative participation of the L-type pentose cycle in hepatic tissues. The studies include liver tissues which are actively proliferating, i.e. foetal, regenerating and neoplastic hepatic tissues, as a test of the proposition that pentose pathway flux is related to cell growth and proliferation.

Investigations *in vitro* using enzyme preparations from normal adult, 17-18 day foetal, and 24h and 48h post-operative regenerating livers and two transplantable

Morris hepatomas (5123C and 7777) were performed to measure any alternations in the capacity for pentose pathway flux or regulation of flux in these tissues. Measurement of the maximum catalytic capacities of the conventional enzymes of the pathway showed that the only consistent alteration in the 'growth' tissues was an elevated activity of glucose 6-phosphate dehydrogenase. Measurements of the tissue concentrations of the intermediates of the pathway and analysis of the data in terms of mass action ratios showed that only the glucose 6-phosphate dehydrogenase catalyzed reaction is far removed from equilibrium in all tissues examined. Thus glucose 6-phosphate dehydrogenase is considered to be the major regulatory site controlling the flux of glucose carbon through the pentose pathway.

The metabolism of ribose 5-phosphate by enzyme preparations was studied in detail over a 2h time-course. The rate of ribose 5-phosphate dissimilation and of hexose 6-phosphate formation showed no growth-related or malignancy-linked trends. It is considered that the capacity of the enzymes from all tissues for the metabolism of ribose 5-phosphate is not significantly altered in 'growth' tissues. Carbon balance studies following the time-course of ribose 5-phosphate metabolism revealed that as much as 10-20% of the substrate carbon could not be accounted for in the intermediates of the F-type pentose pathway. This data suggests that the "missing" carbon may be found in the intermediates of the L-type pentose pathway as was the case for normal adult liver (Williams *et al.*, 1978b).

The metabolism of D-*glycero*-D-*ido*-octulose 1,8

bisphosphate and the rate of hexose 6-phosphate formation from this substrate plus *altro*-heptulose 7-phosphate were investigated. D-*glycero*-D-*ido*-octulose 1,8 bisphosphate is considered to be a diagnostic substrate marker for the operation of the L-type pentose pathway in tissues. Enzyme preparations from all tissues examined were capable of utilizing these substrates for the formation of hexose 6-phosphate.

The metabolism of [1-$^{14}$C] and [6-$^{14}$C] glucose by isolated cells from normal liver, 24h regenerating liver, and hepatoma 5123TC cells was studied by examining the rate of incorporation of $^{14}$C-isotope into $CO_2$, lipid, nucleic acids and protein. The interpretation of these results is limited because of the difference in glycogen content of these cells and the activity of $^{14}$C-exchange reactions. The fate of $^{14}$C in the hepatoma cells indicates a decreased activity of the tricarboxylic acid cycle, increased glycolytic flux and a redirection of glucose carbon into lipid, nucleic acid and protein synthesis relative to normal liver. The metabolism of $^{14}$C-labelled glucose by 24h regenerating liver cells shows that there is an increased rate of glucose conversion to nucleic acids relative to normal liver but no alteration in lipid synthesis from glucose. There is also a decrease in the rate of protein synthesis. Analysis of the relative incorporation of $^{14}$C into cell products from [1-$^{14}$C] glucose compared to [6-$^{14}$C] glucose indicates that there is little change or at most only a moderate increase in the flux of carbon through the oxidative segment of the pentose pathway in regenerating liver compared with normal liver. The

incorporation of $^{14}C$ from [1-$^{14}C$] and [6-$^{14}C$] glucose into nucleic acids is considered to reflect extensive operation of $^{14}C$-exchange reactions in all cell types examined.

The quantitative participation of the pentose pathway to the metabolism of glucose by these tissues cannot be assessed based on studies using [1-$^{14}C$] and [6-$^{14}C$] glucose. A new theoretical approach for assessing the quantitative participation of the L-type pentose cycle is derived. This approach is based on the unique redistribution of $^{14}C$ in the carbon atoms of glucose 6-phosphate following the metabolism of [5-$^{14}C$] glucose by the L-type reaction sequence. Experimentally the estimate involves the application of [5-$^{14}C$] and [2-$^{14}C$] glucose to the test tissue followed by the isolation and degradation of glucose 6-phosphate to determine the relative distribution of $^{14}C$ in each of the 6 carbon atoms.

Analysis of the $^{14}C$ distribution in the carbon atoms of glucose 6-phosphate following the metabolism of [2-$^{14}C$], [5-$^{14}C$] and [4,5,6-$^{14}C$] glucose by isolated cells from normal liver confirmed the operation of the L-type pentose cycle in intact liver cells and also confirmed a number of the assumptions involved in the derivation of expressions for estimating the L-type pentose cycle. The quantitative contribution of the L-type cycle in normal hepatocytes is calculated to be 22-30% of total glucose metabolism. Application of this experimental approach to cells isolated from 24h regenerating liver gave similar qualitative results but showed that only 11% of total glucose metabolism is *via* the L-type pentose cycle. The isotope distribution in the carbon atoms of glucose

6-phosphate isolated from 5123TC cells following the metabolism of [5-$^{14}$C] glucose yielded a similar result to that for normal liver cells, however the distribution following [2-$^{14}$C] glucose metabolism indicated extensive operation of particular $^{14}$C-isotope exchange reactions and the contribution of the L-type pentose cycle in these cells cannot be estimated.

The results of these studies demonstrate that the L-type pentose cycle is the major variant of the pathway in all hepatic tissues examined. The proposed correlation between cellular proliferation and increased pentose pathway flux is not confirmed. It is considered that selective activation of the pentose phosphate pathway is not a necessary prerequisit for cell growth and proliferation.

## NOMENCLATURE AND ABBREVIATIONS

1. Trivial names for sugars and sugar phosphates have been used when there is no possibility of ambiguity. In all other instances systematic names have been employed in accordance with the IUPAC-IUB Commission "Tentative Rules for Carbohydrate Nomenclature" *Biochem J.* (1971) 125, 673.

2. All sugars and sugar phosphates have the D configuration. All keto sugars are 2-keto sugars.

3. The term "exchange reaction" implies exchange between labelled and unlabelled moieties of sugar phosphate molecules in the absence of any net synthesis.

4. Abbreviations in the text, tables and figures conform wherever possible to those of The Biochemical Journal. The following abbreviations have been used:

| | |
|---|---|
| Tri-P | glyceraldehyde 3-P plus dihydroxyacetone-P |
| anthrone | 9,10-dihydro-9-oxoanthracene |
| orcinol | 3,5-dihydroxytoluene |
| -P | phosphate |
| SEM | standard error of mean |

| | |
|---|---|
| EC | Enzyme Commission |
| C-1 .... C-6 | e.g. carbon atoms 1 to 6 of glucose 6-P |
| 2C, 3C | two carbon and a three carbon unit |
| µCi | micro curie ($2.22 \times 10^6$ d.p.m.) |
| µl | micro litre |
| PC | pentose cycle |
| $K_i$ | dissociation constant of inhibitor enzyme complex |
| d.p.m. | disintegrations per min. |
| o.p.m. | oscillations per min. |
| M | molarity |
| mg | milligram |
| nmoles | nanomoles |
| w/v | weight for volume |
| g | gravity |

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# CHAPTER 1

## INTRODUCTION

### 1.1 The pentose phosphate pathway and its role in metabolism

The pentose phosphate pathway or hexose monophosphate shunt comprises reaction sequences which are of major importance in all living organisms. The pathway consists of two parts; an oxidative segment in which glucose 6-phosphate is oxidatively decarboxylated to form ribulose 5-phosphate *via* 6-phosphogluconate (Horecker *et al.* 1951), and a non-oxidative segment, in which pentose 5-phosphates are transformed to hexose 6-phosphates and triose-phosphate. In the oxidative segment of the pathway, which is essentially irreversible, 1.0 mol of glucose 6-phosphate is converted to 1.0 mol of ribulose 5-phosphate with the production of 1.0 mol of $CO_2$ and 2.0 mol of NADPH (reactions 1.1 and 1.3). The ribulose 5-phosphate is converted to ribose 5-phosphate and xylulose 5-phosphate by specific isomerase and epimerase enzymes respectively yielding a pool of pentose 5-phosphate. The pentose 5-phosphate has two alternative fates; (i) as ribose 5-phosphate it may be utilized *via* phosphoribosylpyrophosphate for nucleotide and nucleic acid synthesis or (ii) it may proceed through a series of reactions involving 3C, 4C, 7C, and in liver 8C sugar phosphates to be converted to triose-phosphate and hexose 6-phosphates. Hexose 6-phosphates are known to be products of ribose metabolism in extracts of a number of plant and animal tissues and micro-organisms, e.g.

erythrocytes (Dische, 1938), liver (Schlenk and Waldvogel, 1946), spinach and peas (Axelrod *et al.*, 1953), *Escherichia coli* (Racker, 1948) and yeast (Sable, 1952). The series of reactions leading to the formation of hexose 6-phosphates is referred to as the non-oxidative segment of the pentose pathway. The concerted action of the oxidative and non-oxidative segments of the pathway result in metabolism which may be represented as: Glucose 6-P ⟶ Pentose 5-P ⟶ Glucose 6-P, and therefore constitute a cycle, hence the term pentose phosphate cycle.

Two main functions of the pathway or cycle are well recognized, these are: (i) the synthesis of 3C, 4C, 5C, 7C and 8C sugar phosphate intermediates and (ii) the generation of reduced NADP. The 3C sugar phosphates (glyceraldehyde 3-phosphate and dihydroxyacetone phosphate) formed *via* the pentose pathway may be used for carbohydrate synthesis as in the case of the carbon reduction cycle (Racker, 1957), or be metabolized in the lower half of the glycolytic pathway and the tricarboxylic acid cycle to yield ATP and by-pass the energy-requiring and regulatory enzyme phosphofructokinase (EC 2.7.1.11). Erythrose 4-phosphate is used for the synthesis of aromatic amino acids *via* shikimic acid (Neish, 1960) in bacteria and some plants. The pentose 5-phosphate may be utilized for the synthesis of nucleic acids, purine, pyrimidine, flavin and nicotinamide coenzymes (Cohen, 1947) and ribulose 1,5 bisphosphate, the $CO_2$ acceptor in photosynthesis. NADPH represents the form of metabolic hydrogen required for synthetic and growth processes. The metabolic importance of NADPH is illustrated by the variety and number of reductive

reactions which are NADPH-specific as shown in Table 1.1. Of the six known reactions for the production of NADPH in the cell [reactions catalyzed by glucose 6-phosphate dehydrogenase (EC 1.1.1.49), 6-phosphogluconate dehydrogenase (EC 1.1.1.44), malate dehydrogenase (decarboxylating) (NADP) (EC 1.1.1.40), cytoplasmic isocitrate dehydrogenase (EC 1.1.1.42), cytoplasmic transhydrogenase and the energy linked mitochondrial transhydrogenase], the reactions of the oxidative segment of the pentose pathway are of considerable importance and may be quantitatively the most significant. The involvement for example of the reactions of the oxidative segment of the pentose pathway in hepatic fatty acid synthesis has been inferred from the work of Eger-Neufeldt *et al.* (1965) and Taketa and Pogell (1966) where the synthesis of long chain fatty acyl-SCoA's has been demonstrated to be regulated by "feedback" inhibitory processes at acetyl-SCoA carboxylase (EC 6.4.1.2) and glucose 6-phosphate dehydrogenase (Williams *et al.* 1974b).

It is generally held that the pentose phosphate pathway is a biosynthetic or anabolic pathway of glucose metabolism in tissues and that its major physiological role is the provision of NADPH for reductive biosynthesis and of pentose 5-phosphate for nucleic acid and nucleotide synthesis. However, analysis of the activity of the pentose cycle in growing cells has failed to establish any relationship between cell growth and division and the pentose cycle (Katz and Rognstadt, 1967). Recently an alternative mechanism for the non-oxidative segment of the pentose pathway in liver has been proposed (Williams *et al.*, 1978 a,b; see also section 1.2.2). As a consequence of

this discovery, it was considered pertinent to re-examine the activity of the pentose cycle in relation to hepatic tissue growth. It is this general consideration which has determined the direction of the work described in this thesis. Because the experimental work and the interpretation of results discussed here rests heavily on a particular view of the sequence of reactions comprising the pentose cycle in liver, the historical background of the pathway is treated in detail in the following section.

Table 1.1

Reductive biosyntheses in the mammal which are NADPH-specific (from Williams and Clark, 1971)

| Enzyme | Product of reaction | On pathway leading to | Comments |
|---|---|---|---|
| β - Ketoacyl CoA reductase | β - hydroxy acyl CoA | fatty acids | substrate preference is NADPH: NADH of 6:1 |
| 2,3 dehydro acyl reductase | fatty acyl CoA | fatty acids | as above |
| Palmityl CoA reductase | palmitaldehyde | sphingosine | |
| Phenylalanine hydroxylase | tyrosine | proteins | other aerobic hydroxy-lations are also NADPH specific |
| Steroid hydroxylase | (e.g.) corticosterone | various steroids | |
| Fatty acid hydroxylase | hydroxy-fatty acids | unsaturated fatty acids | enzyme favours NADP |
| Glucose reductase | sorbitol | seminal fructose | pathway acts in direction of fructose synthesis |
| Ring closure and steroid demethylation enzymes | steroids | steroids | NADH is much less effective than NADPH |
| Dihydrofolate reductase | tetrahydrofolate | | |
| Malic enzyme | malate | hexose from pyruvate | |
| Glucuronate reductase | L-glururonate | L-xylulose | |
| Glutathione reductase | glutathione | | |

## 1.2 Historical development of the pentose phosphate pathway

### 1.2.1 The oxidative segment of the pentose pathway

Evidence for the existance of an alternative route for the breakdown of glucose other than the Embden-Meyerhof pathway was first provided by Warburg and Christian (1931, 1937) and Warburg *et al.* (1935) who showed that glucose 6-phosphate was oxidized to 6-phosphogluconolactone (reaction 1.1) and that the 6-phosphogluconate formed by the hydration of the lactone (reaction 1.2) was oxidatively decarboxylated to form a pentose 5-phosphate later identified as ribulose 5-phosphate (reaction 1.3).

$$\text{Glucose 6-phosphate} + NADP^+ \longrightarrow \text{6-phosphogluconolactone} + NADPH + H^+ \quad (1.1)$$

$$\text{6-phosphogluconolactone} + H_2O \longrightarrow \text{6-phosphogluconic acid} \quad (1.2)$$

$$\text{6-phosphogluconic acid} + NADP^+ \longrightarrow CO_2 + \text{ribulose 5-phosphate} + NADPH + H^+ \quad (1.3)$$

Lipman (1936) found that an extract of yeast continued to respire in the presence of bromoacetate, although fermentation is blocked by this substance. This observation was contrary to the concept that respiration necessarily followed a preceding anaerobic glycolysis, and added further evidence for the presence of an alternative path of metabolism from glucose 6-phosphate. Reactions 1.1 and 1.3 were distinguished in this early stage of the history of metabolic pathways by the demonstration of an absolute requirement for NADP, a coenzyme different to that

required for oxidation in glycolysis. From the time of its discovery, NADP was considered to be the coenzyme of respiration and it was this proposed function of NADP which led to an intensive investigation of glucose 6-phosphate oxidation. Although the role of NADP in respiration was soon abandoned following the demonstration by Lehninger (1951) that the coenzyme of oxidative phosphorylation was NAD, the interest of a number of investigators in the oxidative decarboxylation of glucose 6-phosphate was firmly established including that of Horecker and co-workers who were to be instrumental in elucidating the mechanism of the pentose phosphate pathway.

Reactions 1.1 and 1.3 are catalyzed by the enzymes glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase respectively. These enzymes were shown to be widely distributed in mammalian tissues (Dickens and Glock, 1950, 1951; Horecker and Smyrniotis, 1951), in a variety of lower plants and animals (Cohen, 1950) and also in higher plants (Conn and Vennesland, 1951; Gibbs, 1952). Glock and McLean (1953) established the first satisfactory procedures for measuring the maximum catalytic capacity of these proteins and reported a number of kinetic parameters of the enzymes. It is generally agreed that glucose 6-phosphate dehydrogenase is the regulatory enzyme of the oxidative segment of the pentose pathway and that regulation is achieved by adaptive changes in active enzyme concentration and by 'fine' control of enzyme activity by regulatory effector molecules. The enzyme has been extensively studied from a number of sources and a review of its structural, catalytic and regulatory features has recently

appeared (Levy, 1979). The adaptive control of glucose 6-phosphate dehydrogenase is illustrated by the observation that the maximum catalytic capacity of this enzyme increases up to 10 fold in response to a diet containing excess carbohydrate (Tepperman and Tepperman, 1958; Fitch *et al.*, 1959; Fitch and Chaikoff, 1960). A similar 10 fold change in the activity of this enzyme in mouse liver was reported in response to dietary manipulation by Hizi and Yagil (1974) however these authors also reported that this large and reversible increase in activity did not involve the synthesis of any new enzyme protein. This observation indicates that mechanisms involving modulations of existing enzyme molecules may need to be considered for the adaptation of this enzyme at least in mouse liver. By far the most important 'fine' control of glucose 6-phosphate dehydrogenase activity is inhibition of the enzyme by NADPH (Negelein and Haas, 1935; Cahill *et al.*, 1958; Gumaa and McLean, 1971). When the ratio of free NADPH to free NADP is greater than 8, inhibition of the enzyme is almost complete (Krebs and Eggleston, 1973). Since in rat liver *in vivo* the calculated ratio of free NADPH to free NADP is of the order of 100 (Veech *et al.*, 1969), the enzyme must be assumed to be almost completely inhibited *in vivo*, unless there are special mechanisms removing the inhibition. Krebs and Eggleston (1973) reported that oxidized glutathione at physiological concentrations is able to counteract the inhibition of the enzyme by NADPH and also demonstrated the requirement for a highly unstable, unidentified co-factor. Rodriguez-Segade *et al.* (1979) have recently described the isolation of a co-factor from rat liver,

apparently a protein of molecular weight 10,000 daltons, which with oxidized glutathione contributes to the de-inhibition of glucose 6-phosphate dehydrogenase activity in the presence of NADPH. Thus the regulation of glucose carbon flux through the oxidative segment of the pentose phosphate pathway is very complex and involves both adaptive changes in active protein concentration and regulation by a de-inhibition mechanism involving NADPH, glutathione and a protein factor.

### 1.2.2 The non-oxidative segment of the pentose phosphate pathway

The mechanism or sequence of reactions of the non-oxidative segment of the pentose phosphate pathway has been the subject of extensive investigation in this laboratory for the past ten years and has resulted in the proposal and confirmation of two different reaction sequences in tissues. Since the investigations described in this thesis were designed in part to elucidate the mechanism of the pathway in various tissues, the evidence supporting the different mechanisms is discussed in some detail.

The first demonstration of a non-oxidative mechanism for pentose 5-phosphate and hexose 6-phosphate interconversion in metabolism came from the observation of Dickens (1938), that yeast extracts could ferment ribose to yield products similar to those derived from hexose fermentation. Dische (1938) also observed that erythrocyte enzymes could catalyze the conversion of ribose (from adenosine) to a mixture of hexose phosphate and triose phosphate. This observation gave rise to the belief that the pentose

underwent cleavage to a 2C and a 3C fragment, but in spite of an intensive search, the 2C fragment was never isolated. The belief was held that hexose phosphate was formed in these systems from the triose phosphate *via* the concerted actions of triose phosphate isomerase (EC 5.3.1.1), aldolase (EC 4.1.2.6) and fructose 1,6 bisphosphatase (EC 3.1.3.11). Dische (1938) clearly showed however, that haemolysates, which produced hexose 6-phosphate from ribose 5-phosphate, completely lacked fructose 1,6 bisphosphatase. The experiments of Dische (1951) and of Glock (1952) cast further doubt on the above mechanism when they demonstrated that more than 60% of the pentose carbon was found in the hexose product, thus indicating that a portion of the hypothetical 2C fragment was utilized for hexose 6-phosphate synthesis. The discovery of the transketolase reaction (Horecker and Smyrniotis, 1952) partly resolved this dilemma. This arose from the finding that a partially purified liver preparation gave rise to *altro* heptulose 7-phosphate from ribose 5-phosphate. Racker *et al*. (1953) reported that they had crystallized an enzyme from yeast of identical function which they called transketolase (EC 2.2.1.1). This enzyme would transfer a 2C fragment from a ketopentose molecule, provided that an appropriate aldehyde acceptor molecule for the "active glycoaldehyde" was present. Ribose 5-phosphate was an efficient acceptor substrate in the reaction and bound the 2C moiety to form *altro* heptulose 7-phosphate. At that time the ketol donor was believed to be ribulose 5-phosphate, but subsequently Srere *et al*. (1955) and Horecker *et al*. (1956) showed that it was in fact xylulose 5-phosphate. Xylulose 5-phosphate is formed

from ribulose 5-phosphate by the enzyme ribulose 5-phosphate 3-epimerase (EC 5.1.3.1).

Transketolase has an absolute requirement for thyamine pyrophosphate for activity (Racker *et al*. 1953) and the enzyme exhibits a broad specificity with respect to donor and acceptor molecules (Racker, 1961 ). The reaction catalyzed by transketolase is readily reversible (Horecker *et al*. 1953), however the glycoaldehyde moiety or 2C fragment is tightly bound to the enzyme *via* thyamine pyrophosphate and is never found "free" in solution (Krampitz *et al*. 1961; Holtzer *et al*. 1962).

The *altro* heptulose 7-phosphate and glyceraldehyde 3-phosphate formed by transketolase are substrates for transaldolase (EC 2.2.1.2) yielding a hexose 6-phosphate as one of the reaction products (Horecker and Smyrniotis, 1953). This is achieved by transfer of a 3C dihydroxyacetone moiety from donor to aldo acceptor, thus forming fructose 6-phosphate and a tetrose-phosphate. The nature of the 4C aldo sugar was predicted with confidence to be erythrose 4-phosphate by inspection of the configuration of the parent heptulose donor. Synthetic erythrose 4-phosphate was also found to be a substrate for transaldolase as shown in reaction 1.4 (Kornberg and Racker, 1955).

Erythrose 4-P + fructose 6-P $\rightleftharpoons$ *altro* heptulose 7-P + glyceraldehyde 3-P (1.4)

Having identified a set of enzymes, cofactors and intermediates which established the non-oxidative segment of the pentose phosphate pathway as a viable alternative pathway of glucose metabolism, it remained only to

establish the reaction sequence and the stoichiometry. The classical textbook mechanism of the non-oxidative segment of the pathway was based on the results of two experiments (Horecker *et al*. 1954; Gibbs and Horecker 1954). These experiments involved the separate incubation of [1-$^{14}$C] ribose 5-phosphate and [2,3-$^{14}$C] ribose 5-phosphate with buffered extracts of acetone dried powders prepared from rat liver, pea root and pea leaf tissues. The incubations were carried out for 16h and 17h with liver, 4h with pea root and 1h and 2h for pea leaf. With liver the [1-$^{14}$C] ribose 5-phosphate gave rise to [1,3-$^{14}$C] glucose 6-phosphate with 74% of the $^{14}$C in C-1 and 24% in C-3. The [2,3-$^{14}$C] ribose 5-phosphate yielded glucose 6-phosphate labelled as follows: 45% in C-4, 28% in C-2, 20% in C-3 and 7% in C-1. The isotope distribution pattern found in the glucose 6-phosphate isolated from the pea root incubation was essentially the same as that for liver while the shorter incubations with pea leaf were significantly different.

It was considered that these distributions of $^{14}$C isotope were explained by reactions sequentially catalyzed by ribose 5-phosphate isomerase (reaction 1.5), ribulose 5-phosphate 3-epimerase (reaction 1.6), followed by transketolase (reaction 1.7), transaldolase (reaction 1.8) and transketolase (reaction 1.9 - see Fig. 1.1) (Horecker *et al*. 1954, Gibbs and Horecker 1954). In the case of [1-$^{14}$C] ribose 5-phosphate the reaction sequence of Fig. 1.1 would theoretically predict isotope incorporation into carbons one and three of glucose 6-phosphate in the ratio 2:1, whereas the experimentally determined value was 3:1. With

these results as the sole evidence, this "tentative" proposal became established as the accepted mechanism of the non-oxidative segment of the pentose phosphate pathway.

The carbon balance studies of Glock (1952) and the mechanistic studies of Horecker *et al.* (1954) have resulted in the proposal that the stoichiometry of the reactions of the non-oxidative segment of the pentose phosphate pathway required 3 mol of pentose 5-phosphate to form 2 mol of fructose 6-phosphate and one mol of glyceraldehyde 3-phosphate (reaction 1.10, Fig. 1.1). A pentose phosphate cycle for glucose metabolism was defined by Wood and Katz (1958), such that fructose 6-phosphate formed in the reactions of the non-oxidative segment was converted into glucose 6-phosphate by glucosephosphate isomerase (EC 5.3.1.9), and the resulting stoichiometry for one turn of the cycle was:

$$\text{Glucose 6-P} + 6\ \text{NADP}^{+} \longrightarrow 3\text{CO}_2 + \text{glyceraldehyde 3-P} + 6\ \text{NADPH} + 6\ \text{H}^{+} \quad (1.11)$$

The enzymatic reactions involved in this pentose phosphate cycle are shown in Fig. 1.2. On the basis of this mechanism of the pentose cycle, Katz and Wood (1960) put forward an elaborate theory for quantitatively measuring the relative contribution of the pentose cycle to the overall metabolism of glucose by tissues. A detailed analysis of this theoretical method is presented elsewhere (see Section 1.3) however essentially the theory involved a mathematical evaluation of the ordered redistribution of either carbons 2 or 3 of substrate glucose into positions 1,2 and 3 of glucose 6-phosphate. The model predicted randomization of

2 [1-$^{14}$C] Ribose 5-P $\rightleftharpoons$ 2 [1-$^{14}$C] ribulose 5-P (1.5)

2 [1-$^{14}$C] Ribulose 5-P $\rightleftharpoons$ 2 [1-$^{14}$C] xylulose 5-P (1.6)

[1-$^{14}$C] Xylulose 5-P + [1-$^{14}$C] ribose 5-P $\rightleftharpoons$ [1,3-$^{14}$C] *altro* heptulose 7-P + Gra-P (1.7)

[1,3-$^{14}$C] *altro* Heptulose 7-P + Gra-P $\rightleftharpoons$ [1,3-$^{14}$C] fructose 6-P + erythrose 4-P (1.8)

[1-$^{14}$C] Xylulose 5-P + erythrose 4-P $\rightleftharpoons$ [1-$^{14}$C] fructose 6-P + Gra-P (1.9)

---

*Sum:* 3 [1-$^{14}$C] Ribose 5-P $\rightleftharpoons$ [1-$^{14}$C] fructose 6-P + [1,3-$^{14}$C] fructose 6-P + Gra-P (1.10)

Fig. 1.1 Series of reactions proposed by Horecker *et al.* (1954) to explain the distribution of $^{14}$C in glucose 6-P formed from [1-$^{14}$C] ribose 5-P, in sequences catalyzed by enzyme from rat liver

$^{14}C$ from [2-$^{14}C$] glucose into positions 1 and 3 of glucose 6-phosphate *via* the reactions of the pentose cycle as shown in Fig. 1.2. Theoretically such redistribution is the exclusive property of the pentose cycle and the degree of redistribution of radioactivity following the metabolism of [2-$^{14}C$] glucose measures the quantitative contribution of the cycle relative to total glucose metabolized.

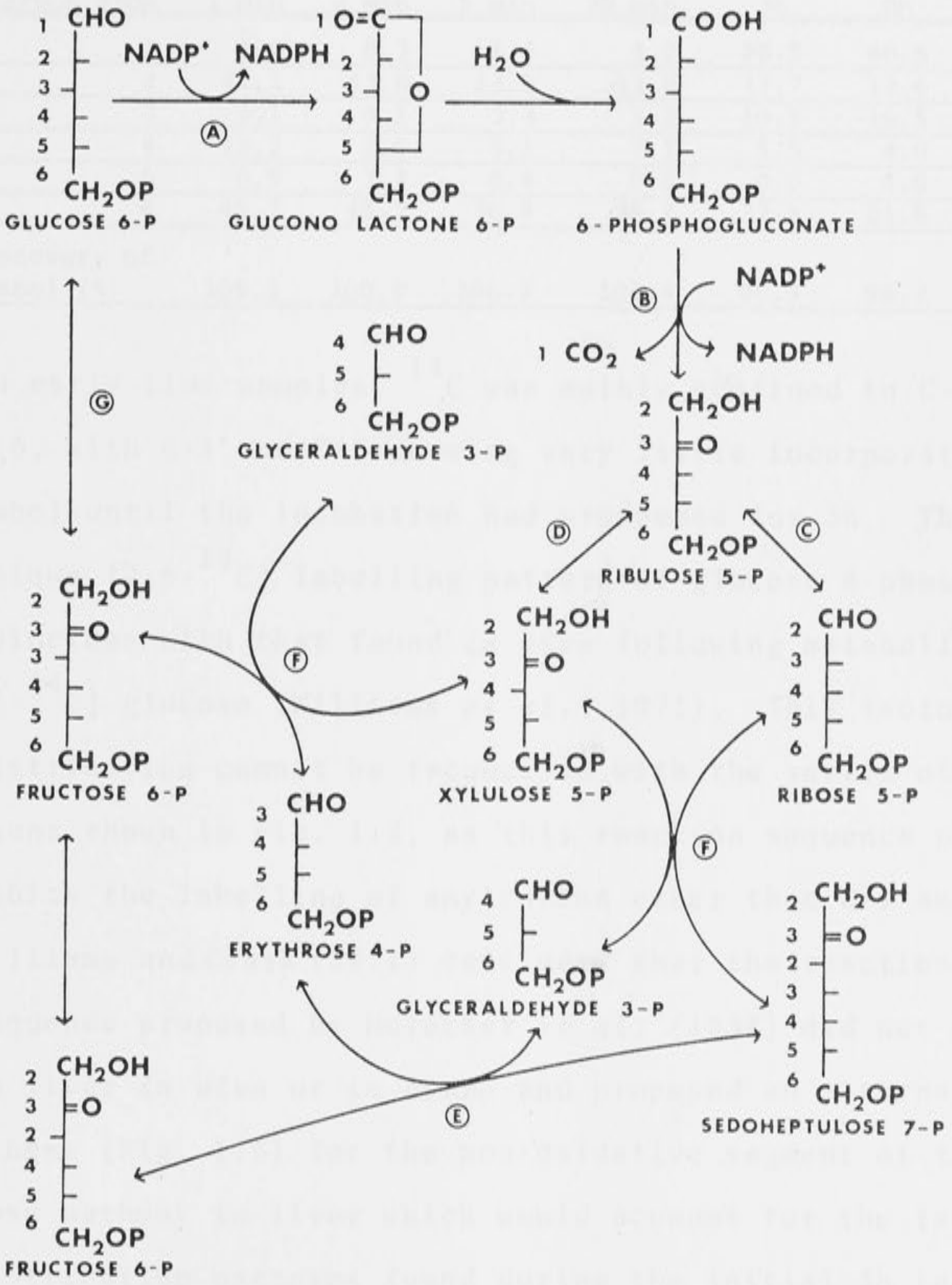
Williams (1966) and Williams *et al*. (1971) studied the metabolism of [2-$^{14}C$] glucose and [1-$^{14}C$] ribose in rabbit liver *in vivo*, in attempts to quantitatively measure the contribution of the pentose cycle to glucose metabolism by the method of Katz and Wood (1960). The expected isotope distribution in positions 1 and 3 of glucose 6-phosphate did not occur, but instead C-2 and C-6 of glucose 6-phosphate were heavily labelled. It was concluded that the isotope distribution patterns could not be reconciled with the carbon transferring mechanisms of any then known pathway of carbohydrate metabolism in animal tissue. The application of [2-$^{14}C$] glucose to the isolated perfused liver (Schofield *et al*. 1970) yielded a similar result. The results of these studies caused Williams and co-workers to question the validity of the Katz and Wood (1960) treatment and particularly to question the reaction scheme depicted in Fig. 1.2 in liver. This led to a reinvestigation of the definitive experiment of Horecker *et al*. (1954).

On repeating and extending the original Horecker *et al*. (1954) experiments, Williams and Clark (1971) found that the $^{14}C$ distribution patterns in glucose 6-phosphate formed by reactions of [1-$^{14}C$] ribose 5-phosphate with

Fig. 1.2: Reaction sequence of the F-type pentose phosphate cycle

The sequence of reactions comprising the pentose phosphate pathway in adipose tissue as proposed by Horecker *et al.* (1954) is shown. The numbers to the left of the structural formulae of each of the intermediates represent the carbon atoms of the substrate glucose. The enzymes involved in the cycle are: (A) glucose 6-phosphate dehydrogenase, (B) 6 phosphogluconate dehydrogenase, (C) ribose 5-phosphate isomerase, (D) ribulose 5-phosphate 3-epimerase, (E) transaldolase, (F) transketolase, (G) glucosephosphate isomerase.

FIG 1.2


GLUCOSE 6-P
NADP+
NADPH
A
GLUCONO LACTONE 6-P
H2O
6-PHOSPHOGLUCONATE
B
NADP+
CO2
NADPH
RIBULOSE 5-P
D
C
XYLULOSE 5-P
RIBOSE 5-P
GLYCERALDEHYDE 3-P
G
F
FRUCTOSE 6-P
ERYTHROSE 4-P
GLYCERALDEHYDE 3-P
F
SEDOHEPTULOSE 7-P
E
FRUCTOSE 6-P

enzymes of rat liver were markedly different at early time periods from those seen after 17h of incubation (Table 1.2).

Table 1.2 Percentage distribution of $^{14}C$ in glucose 6-phosphate formed by reactions of [1-$^{14}C$] ribose 5-phosphate with enzymes of rat liver. (Williams and Clark, 1971).

| Carbon atom | 1 min | 2 min | 5 min | 30 min | 3h | 8h | 17h |
|---|---|---|---|---|---|---|---|
| 1 | 1.4 | 0.7 | 1.3 | 1.2 | 28.5 | 40.5 | 56.5 |
| 2 | 44.1 | 43.8 | 13.0 | 11.8 | 17.7 | 12.6 | 12.4 |
| 3 | 1.1 | 0.1 | 3.3 | 1.1 | 10.5 | 16.5 | 24.4 |
| 4 | 12.2 | 2.6 | 5.1 | 7.1 | 5.5 | 4.0 | 2.0 |
| 5 | 0.5 | 7.1 | 0.4 | 0.6 | 0.0 | 4.6 | 1.6 |
| 6 | 40.7 | 45.7 | 76.9 | 78.2 | 37.8 | 21.8 | 3.1 |
| Recovery of label (%) | 105.1 | 100.8 | 106.2 | 103.4 | 96.9 | 98.7 | 100.5 |

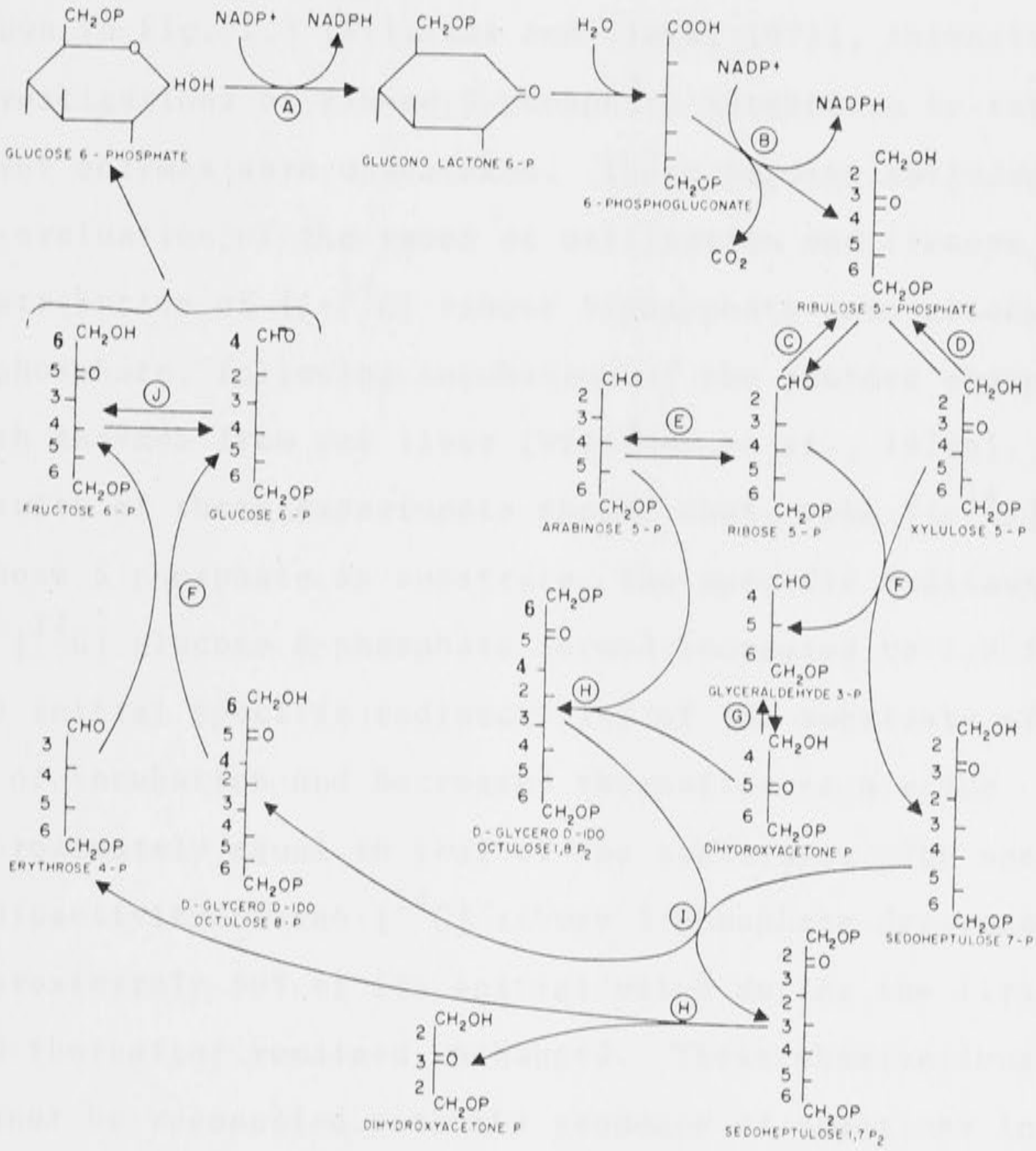
In early time samples, $^{14}C$ was mainly confined to C-2 and C-6, with C-1 and C-3 showing very little incorporation of label until the incubation had proceeded for 3h. This unique [2,6-$^{14}C$] labelling pattern of glucose 6-phosphate coincides with that found *in vivo* following metabolism of [2-$^{14}C$] glucose (Williams *et al.*, 1971). This isotope distribution cannot be reconciled with the series of reactions shown in Fig. 1.2, as this reaction sequence prohibits the labelling of any carbon other than C-1 and C-3. Williams and Clark (1971) concluded that the reaction sequence proposed by Horecker *et al.* (1954) did not occur in liver *in vivo* or *in vitro* and proposed an alternative scheme (Fig. 1.3) for the non-oxidative segment of the pentose pathway in liver which would account for the isotope distribution patterns found during the initial 3h incubation period.

The scheme of Fig. 1.3 has a number of distinguishing features, including four additional intermediates (arabinose 5-phosphate, *altro*-heptulose 1,7-bisphosphate, D-*glycero*-D-*ido*-octulose 8-phosphate and D-*glycero*-D-*ido*-

Fig. 1.3: Redistribution of the carbon atoms of glucose by the L-type pentose phosphate cycle

The sequence of reactions comprising the pentose phosphate cycle in liver as proposed by Williams *et al*. (1978b) is shown. The carbon atoms of the original glucose are indicated to the left of the structural formulae of each of the intermediates and the numbers to the right of the triose phosphates represent the carbon atoms of that molecule. The enzymes involved in the cycle are indicated by the letters and are: (A) glucose 6-phosphate dehydrogenase; (B) 6-phosphogluconate dehydrogenase; (C) ribose 5-phosphate isomerase; (D) ribulose 5-phosphate 3 epimerase; (E) arabinose 5-phosphate epimerase; (F) transketolase; (G) triosephosphate isomerase; (EC 5.3.1.1); (H) aldolase; (EC 4.1.2.13); (I) D-*glycero*-D-*ido*-octulose 1,8 bisphosphate : D-*altro*-heptulose 7-phosphotransferase; (J) glucosephosphate isomerase.

FIG 1.3


GLUCOSE 6 - PHOSPHATE
NADP+
NADPH
A
GLUCONO LACTONE 6-P
H2O
COOH
NADP+
NADPH
B
6 - PHOSPHOGLUCONATE
CO2
RIBULOSE 5 - PHOSPHATE
C
D
ARABINOSE 5 - P
RIBOSE 5 - P
XYLULOSE 5 - P
E
F
J
FRUCTOSE 6 - P
GLUCOSE 6 - P
GLYCERALDEHYDE 3-P
G
DIHYDROXYACETONE P
H
D - GLYCERO D - IDO OCTULOSE 1,8 P2
ERYTHROSE 4 - P
D - GLYCERO D - IDO OCTULOSE 8 - P
I
SEDOHEPTULOSE 7 - P
DIHYDROXYACETONE P
SEDOHEPTULOSE 1,7 P2

octulose 1,8-bisphosphate) and two new enzymes (arabinose 5-phosphate 2-epimerase and phosphotransferase). The reaction sequence of Fig. 1.3 does not include a transaldolase-catalysed reaction, but it preserves the stoichiometry of the pathway of Horecker *et al*. (1954) (Equation 1.10).

Following the proposal of the reaction sequence shown in Fig. 1.3 (Williams and Clark, 1971), intensive investigations of ribose 5-phosphate metabolism by rat liver enzymes were undertaken. These studies included a re-evaluation of the rates of utilization and isotope re-distribution of [1-$^{14}$C] ribose 5-phosphate into glucose 6-phosphate, following incubation of the pentose phosphate with enzymes from rat liver (Williams *et al*., 1978a). The results of these experiments showed that, with [1-$^{14}$C] ribose 5-phosphate as substrate, the specific radioactivity of [$^{14}$C] glucose 6-phosphate formed increased to 1.9 fold the initial specific radioactivity of the substrate after 3h of incubation and decreased thereafter to a value approximately equal to that of the substrate. The specific radioactivity of the [$^{14}$C] ribose 5-phosphate decreased to approximately 50% of its initial value during the first 3h and thereafter remained unchanged. These observations cannot be reconciled with the sequence of reactions in Fig. 1.2, however the decline in the specific radioactivity of ribose 5-phosphate can be explained by the reactions of Fig. 1.3 and an alternative transketolase-catalyzed reaction (Williams *et al*., 1978b).

It is to be noted that the reaction scheme of Fig. 1.3 was proposed on purely theoretical grounds and the only justification for such a scheme was its ability to explain

the heavy labelling of C-2 of glucose 6-phosphate from [1-$^{14}$C] ribose 5-phosphate (Table 1.2). The possible role of octulose phosphates in the pathway was prompted by the report of Bartlett (1968) that these 8C sugar phosphates are found in erythrocytes. On the basis of this theoretical pathway mechanism, experiments were designed to confirm the metabolic role of the 'new' intermediates in liver. Clark (1972) succeeded in synthesizing D-*glycero*-D-*ido*-octulose 1,8 bisphosphate using the aldolase catalyzed condensation of arabinose 5-phosphate with dihydroxyacetone phosphate and further demonstrated the formation of hexose 6-phosphate when this 8C sugar phosphate was incubated with sedoheptulose 7-phosphate and a rat liver enzyme preparation (Williams *et al.*, 1978b). The formation of triose phosphates and hexose 6-phosphates by rat liver enzymes from arabinose 5-phosphate was also shown (Clark, 1972) and reported by Williams *et al.* (1978b). The results of these experiments indicated that these two 'new' compounds could act as intermediates in the formation of hexose 6-phosphate from pentose 5-phosphate and prompted a detailed study of the intermediates formed from ribose 5-phosphate by rat liver enzymes. Preliminary carbon balance studies indicated that as much as 20% of substrate carbon could not be accounted for when only the intermediates of the reaction sequence of Fig. 1.2 were measured (Blackmore, 1973) and reported by Williams *et al.* (1978b). As a consequence of this observation, a chromatographic examination of all the intermediates formed in the conversion of ribose 5-phosphate to hexose 6-phosphate was conducted. Five additional intermediates of pentose 5-phosphate metabolism in liver were detected, namely

D-*manno*-heptulose 7-phosphate, D-*altro*-heptulose 1,7-bisphosphate, D-*glycero*-D-*ido*-octulose 1,8-bisphosphate, D-*glycero*-D-*altro*-octulose 1,8-bisphosphate and D-arabinose 5-phosphate (Williams *et al.*, 1978b). A re-evaluation of the carbon balance data, including the concentrations of the five new intermediates, revealed that more than 97% of the substrate carbon was accounted for at all times examined during the incubation period. In short, the complete analysis of the reactants, intermediates and products of ribose 5-phosphate metabolism by liver enzymes demonstrated the presence of those intermediates proposed by Williams and Clark (1971) and shown in Fig. 1.3. The intermediates, *altro* and *ido* octulose phosphates, *altro* heptulose 1,7 bisphosphate and *manno*-heptulose 7-phosphate have also been found and measured in fresh liver tissue (Paoletti *et al.*, 1979a).

The experimental findings briefly outlined above support the operation of a new form of the pentose cycle as illustrated in Fig. 1.3 in liver tissue. Preliminary evidence also suggests that the reaction scheme in Fig. 1.3 operates in heart and other tissues. This reaction sequence will: (1) account for the various distributions of $^{14}C$ in glucose 6-phosphate from [1-$^{14}C$] ribose 5-phosphate; (2) account for the failure to measure by the method of Katz and Wood (1960) a quantitatively significant contribution of the pentose phosphate pathway to liver metabolism, in spite of the presence of high activities of the enzymes conventionally assigned to the pathway (Novello and McLean, 1968) and (3) afford a more reasoned and satisfactory explanation of the anomalous $^{14}C$-labelling patterns found

by Williams *et al.* (1971) in hexose 6-phosphates after metabolism of [2-$^{14}$C] glucose and [1-$^{14}$C] ribose in liver *in situ*.

### 1.2.3 The current status of the pentose phosphate cycle in liver and other tissues.

At least two reaction sequences for the pentose phosphate cycle of glucose metabolism are found in tissues. The first of these, shown in Fig. 1.2, is the classical pathway which has been detailed in textbooks of biochemistry since 1956. The reaction sequence of Fig. 1.2 is hereafter referred to as the fat cell or F-type pathway since it has been established that this mechanism exclusively operates in rat epididymal fat tissue (Williams *et al.*, 1974b). The second reaction sequence was found in liver (Williams *et al.*, 1978a,b) and its existance in photosynthetic tissue was proposed by Clark *et al.* (1974). This reaction sequence, (Fig. 1.3), is referred to as the L-type (liver) pentose phosphate cycle. The reactions of both the F-type and L-type pathways have the same overall stoichiometry which is that of equation 1.11. As previously discussed (Section 1.2.1), the enzymes of the pentose pathway are widely distributed in mammalian tissues. The contribution of the pentose pathway to glucose metabolism is considered to be very large in lactating mammary gland (McLean 1960), lung (O'Neil and Tierney 1974) and activated macrophages (Rossi *et al.*, 1972) however it is not known which pathway mechanism operates in these tissues. This is more than an academic point since methods commonly employed for estimating the quantitative participation of the pentose cycle to

the total metabolism of glucose by tissues are dependent upon the pathway reaction sequence. This issue is treated in the following section.

## 1.3 Quantitative measurements of the pentose cycle in liver and other tissues

### 1.3.1 The method of Katz and Wood (1960)

A number of sophisticated methods have been developed for estimating the quantitative contribution of the F-type pentose cycle to glucose metabolism in tissues (Wood and Katz, 1958; Katz and Wood, 1960; Wood *et al.*, 1963; Katz and Wood, 1963; Landau *et al.*, 1964; Landau and Katz, 1965; Katz and Rognstad, 1967; Landau and Bartsch, 1966; Katz and Wals, 1972). The quintessence of all the above methods involved the application of mathematical expression/s describing the rhythmical and ordered redistribution of either carbons 2 or 3 of substrate glucose into positions 1,2 and 3 of glucose 6-phosphate. More specifically, the method of Katz and Wood (1960) involves the application of either [2-$^{14}$C] or [3-$^{14}$C] glucose to the test tissue and, after attainment of isotopic steady state between the fructose 6-phosphate generated from the non-oxidative segment of the pathway and the pools of fructose 6-phosphate and glucose 6-phosphate (Landau *et al.*, 1964), either glucose 6-phosphate or fructose 6-phosphate should be isolated and the specific radioactivities of C-1, C-2 and C-3 determined. From the ratios of the specific radioactivity of C-1/C-2 or C-3/C-2, an estimate of the relative contribution of the pentose phosphate cycle to glucose metabolism can be calculated (Katz and Wood, 1960; Wood

*et al.*, 1963; Landau and Katz, 1965). A detailed treatment of the mathematical procedure of the method of Katz and Wood (1960) is given by Williams and Clark (1971) and is discussed with alterations in Chapter 5 of this thesis.

The theoretical treatment of Katz and Wood is based upon a simplified model of glucose metabolism and of necessity has a number of limitations. Briefly the limitations are:

(i) That the pentose phosphate cycle is complete and is entirely described by the reactions shown in Fig. 1.2.

(ii) The system is in metabolic and isotopic steady state (Landau *et al.*, 1964). The definition of isotopic steady state also applies to conditions under which the specific radioactivity is constant or is changing, e.g. after a single isotope injection *in vivo*.

(iii) The specific radioactivities of glucose 6-phosphate and fructose 6-phosphate should be the same, i.e. the activity of glucose phosphate isomerase should be high and the isomerization rate rapid.

(iv) There is no randomization of the carbon atoms of hexose 6-phosphate by reactions other than those of the F-type pentose phosphate cycle. Examples of such randomizing reactions are the transketolase exchange reaction (Clark *et al.*, 1971), the transaldolase exchange reaction (Ljungdahl *et al.*, 1961) and a coupled transketolase-transaldolase exchange reaction (Wood and Katz, 1958).

(v) There is no synthesis of hexose or hexose derivatives by reversal of the Embden-Meyerhof pathway, thus causing dilution of the specific radioactivity of the substrate glucose by reactions other than the pentose cycle.

(vi) The net equation of the pentose cycle shows the synthesis of one molecule of glyceraldehyde 3-phosphate which is derived from C-4, C-5 and C-6 of the original glucose substrate. There are two molecules formed in the pentose cycle, one of which is converted to fructose 6-phosphate by transaldolase (reaction 1.8) and is recycled, hence it is possible that the glyceraldehyde 3-phosphate will equilibrate with the triose phosphate of the Embden-Meyerhof pathway. Thus triose phosphate, containing $^{14}C$ from C-1, C-2 and C-3 of substrate glucose, may be introduced into C-4, C-5 and C-6 of fructose 6-phosphate. It is not possible, using radioisotope methods to distinguish this effect from that of the transaldolase exchange reaction mentioned above (iv).

Although these limitations may act to interfere with the clear interpretation of data, the method of Katz and Wood (1960) remains the least qualified approach to the problem of estimating the quantitative participation of the F-type pentose cycle. It is important to emphasize that the method of Katz and Wood (1960) will not allow for estimation of the L-type pentose cycle since the carbon redistributions of this pathway are more complex.

1.3.2 A brief summary of the methods for estimating the quantitative participation of the pentose cycle to glucose metabolism

Although the method of Katz and Wood (1960) remains the most acceptable approach for estimating the activity of the F-type pentose cycle, experimentally it is cumbersome and time-consuming since it involves the isolation of glucose 6-phosphate and its carbon by carbon degradation. Consequently a number of alternative and experimentally more simple approaches have been proposed. These methods are discussed below in terms of their applicability to measurement of the L-type pentose cycle.

Experimentally the most convenient method for estimating the quantitative contribution of the pentose cycle to glucose metabolism involves the use of [1-$^{14}$C] and [6-$^{14}$C] glucose and the measurement of the specific yield of $^{14}$C-isotope in $CO_2$ (Katz and Wood, 1963). The theoretical basis of this method depends on the assumption that [1-$^{14}$C] glucose will be metabolized *via* the oxidative segment of the pentose cycle to yield $^{14}CO_2$ and unlabelled pentose 5-phosphate. The metabolism of [6-$^{14}$C] glucose *via* the pentose cycle, however, will not yield $^{14}CO_2$ and the pentose 5-phosphate will be isotopically labelled. Further, both [1-$^{14}$C] and [6-$^{14}$C] glucose will yield [3-$^{14}$C] triose phosphate *via* the glycolytic pathway (assuming isotopic equilibration of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate) and thus will produce $^{14}CO_2$ in equivalent amounts *via* the tricarboxylic acid cycle. Thus in a tissue with an active pentose pathway, [1-$^{14}$C] glucose should produce a greater yield of $^{14}CO_2$ than [6-$^{14}$C] glucose. Katz

and Wood (1963) derived the following expression for calculating the percentage contribution of the F-type pentose cycle to the metabolism of glucose.

$$\%PC = \frac{S}{3 - 2S} \times 100; \quad S = \frac{G_1CO_2 - G_6CO_2}{1 - G_6CO_2} \qquad (E1.1)$$

The terms of $G_1CO_2$ and $G_6CO_2$ represent the specific yield of $^{14}CO_2$ from [1-$^{14}$C] and [6-$^{14}$C] glucose respectively. The specific yield is defined as the amount of $^{14}$C-isotope recovered in $CO_2$ as a fraction of the utilized glucose. The authors emphasized the necessity to measure specific yields rather than the more usual "ratios" data. C-1/C-6 ratios involve the determination of the ratio of $^{14}$C in $CO_2$ from [1-$^{14}$C] and [6-$^{14}$C] glucose. It is known that the C-1/C-6 ratio depends on three independent variables, (i) the percentage of glucose metabolized by the glycolytic pathway, (ii) the percentage of glucose metabolized *via* the pentose pathway and (iii) the extent of oxidation of C-3 of triose phosphate (Wood and Katz, 1958; Katz, 1961). Thus a particular ratio may correspond to numerous combinations of metabolic pathways. C-1/C-6 ratio data are at best a useful qualitative index of pentose pathway activity. They have no role in the quantitation of the pathway.

In specific yield measurements, the $G_6CO_2$ is a measure of the extent of the oxidation of carbon 3 of triose phosphate and thus the advantageous feature of E1.1 is that it only involves 2 variables, namely the contribution of the pentose cycle and the Embden-Meyerhof pathway. The equation also specifies that these two pathways must operate exclusively (i.e. there is no other pathway of glucose

dissimilation). If in addition glucose is metabolized by nontriose pathways, the estimation of the F-type pentose cycle from specific yields in $CO_2$ cannot be made unless an independent determination of one of the pathways is performed. Thus the application of E1.1 when nontriose pathways are operating will result in a maximum estimate of the percentage contribution of the F-type pentose cycle. The effect of recycling of pentose 5-phosphate to hexose 6-phosphate has also been considered in the derivation of E1.1. This is necessary since there is dilution of $^{14}C$ in the hexose 6-phosphate formed from [1-$^{14}C$] glucose *via* the pentose cycle but a similar dilution does not occur with [6-$^{14}C$] glucose (Katz and Wood, 1963).

Expression 1.1 is based entirely on a model of glucose metabolism in which the pentose cycle of Fig. 1.2 is the only reaction sequence which can direct carbons 1,2 and 3 of glucose into C-1 of glucose 6-phosphate where it is released as $CO_2$ in direct proportion to the contribution of that cycle to glucose metabolism. A corollary of this condition is that carbons 4,5 and 6 of glucose never enter carbon 1 of glucose 6-phosphate. The L-type pentose cycle reactions do admit C-6 of glucose into position 1 of glucose 6-phosphate. Thus expression 1.1 cannot be used to measure the pentose cycle in liver and those other tissues where the reactions depicted in Fig. 1.3 operate.

The basic assumption in the derivation of E1.1 involves the condition that the term $G_6CO_2$ represents the extent of oxidation of C-3 of triose phosphate, however the L-type pentose cycle results in [1-$^{14}C$] glucose 6-phosphate formation from [6-$^{14}C$] glucose (see Fig. 1.3) hence $^{14}CO_2$

will be formed from [6-$^{14}$C] glucose *via* the oxidative segment of the pentose cycle and $G_6CO_2$ will not represent C-3 oxidation *via* the tricarboxylic acid cycle in liver. The same argument applies to the isotope dilution effect in hexose 6-phosphate since E1.1 was derived on the assumption that C-6 of glucose could not yield $^{14}CO_2$ *via* the pentose cycle and therefore all hexose 6-phosphate reformed by recycling is labelled with $^{14}$C-isotope. This assumption is not valid for any hexose 6-phosphate which has passed through more than one turn of the L-type pentose pathway mechanism.

An alternative method for measuring the contribution of the F-type pentose cycle involves the yield of $^{14}$C-isotope in triose phosphate derivatives such as lactate, glycerol or lipid following the metabolism of [1-$^{14}$C] and [6-$^{14}$C] glucose. The theoretical basis of this method relies on the assumption that metabolism of [6-$^{14}$C] glucose will yield $^{14}$C-labelled triose phosphate *via* both the glycolytic and pentose pathways whereas [1-$^{14}$C] glucose will yield $^{14}$C-labelled triose phosphate *via* the glycolytic pathway only. Thus the ratio of $^{14}$C-isotope in triose-phosphate derivatives from [6-$^{14}$C] glucose relative to [1-$^{14}$C] glucose should be greater than 1 in a tissue with an active pentose cycle. The percentage contribution of the pentose cycle and the Embden-Meyerhof pathway can be determined from E1.2 and E1.3 (Katz *et al.*, 1966).

$$\%PC = \frac{1 - \gamma}{1 + 2\gamma}; \quad \text{where } \gamma = \frac{[1\text{-}^{14}C] \text{ glucose into triose derivative}}{[6\text{-}^{14}C] \text{ glucose into triose derivative}} \qquad (E1.2)$$

$$\%PC = \frac{S'}{3 - 2S'}; \quad \text{where } S' = G_1CO_2 - [G_6CO_2 - \gamma] \qquad (E1.3)$$

The two expressions (E1.2 and E1.3) differ in that expression E1.2 is not valid in the absence of isotopic equilibration of triose phosphates and does not require a measurement of glucose utilization whereas E1.3 does not involve the assumption of isotopic equilibration but requires a measurement of glucose utilization for calculation of the $G_1CO_2$ and $G_6CO_2$ terms. It is of note that neither of these expressions provides for metabolism of glucose by nontriose phosphate pathways (i.e. synthesis of glycogen, nucleic acids etc.). Furthermore, neither of these expressions will measure the contribution of the L-type pentose cycle since the central assumption that [6-$^{14}$C] glucose forms $^{14}$C-labelled triose phosphate *via* the pentose cycle is untrue for this reaction sequence. The product triose phosphate of the L-type pentose cycle consists of carbon atoms 2,3 and 2 of the original substrate glucose (Fig. 1.3) and therefore will be unlabelled when [6-$^{14}$C] glucose is substrate. It is noted that two molecules of triose phosphate are formed by the non-oxidative segment of the L-type pentose pathway, one of which consists of carbon atoms 4,5 and 6 of substrate glucose and is used for the resynthesis of hexose 6-phosphate. Equilibration of these two pools of triose-phosphate will result in $^{14}$C-labelled triose-phosphate derivatives from [6-$^{14}$C] glucose *via* the L-type pentose cycle, however even with total equilibration of the two pools the amount of $^{14}$C-isotope in triose phosphate formed *via* the L-type pentose cycle will be only ½ of that formed by the F-type pathway.

The three expressions discussed above represent the most commonly employed experimental approaches for measuring

the contribution of the F-type pentose cycle to glucose metabolism. It is clear that none of these methods will allow an accurate estimate of the L-type pentose cycle and therefore they are quite inappropriate for application to liver tissue. A number of extensions of these methods have been proposed (Landau *et al.*, 1964; Landau and Bartsch, 1966; Katz and Rognstad, 1967) however, each of these are based on the same assumptions as the methods discussed above and for the same reasons do not apply to measurement of the L-type pentose cycle.

### 1.3.3 The contribution of the pentose phosphate cycle to the metabolism of glucose in liver

It has not been possible to reach an agreement about the quantitative significance of the pentose cycle in liver due to the variability of a number of estimates (for a review see Katz, 1961). More recent estimates have ranged from values of 2 to 7% of total glucose metabolism based on *in vivo* studies using the method of Katz and Wood (1960) (Hostetler and Landau, 1967) to 13-17% based on measurements of specific yields of $^{14}C$-isotope in $CO_2$ and lipid following the metabolism of [1-$^{14}C$] and [6-$^{14}C$] glucose by isolated hepatocytes (Baquer *et al.*, 1973). This variability has in large part to be attributed to the use of theoretical expressions for the measurement of the pentose cycle which have assumed that the reaction sequence of the non-oxidative segment of the pathway in liver is that proposed by Horecker *et al.* (1954) and shown in Fig. 1.2. As discussed above (Section 1.3.2), none of the mathematical expressions derived for estimating the F-type pentose cycle

apply to the measurement of the L-type pentose cycle due to the more complex $^{14}C$-isotope redistribution that occurs in the non-oxidative segment of this pathway. A new metabolic theory for estimating the L-type pentose cycle and application to isolated hepatocytes are reported in Chapters 5 and 6 of this thesis.

## 1.4 Metabolism of foetal, regenerating and neoplastic hepatic tissues in relation to cellular proliferation and the role of the pentose phosphate pathway

### 1.4.1 Prefatory comment

The underlying proposition which has determined the direction of the research described in this thesis is that the pentose phosphate pathway is essentially a biosynthetic pathway of glucose metabolism. It was considered therefore that the flux of glucose carbon through the pentose cycle should correlate with those processes accompanying cell growth and division. Foetal, regenerating and neoplastic liver tissues were chosen for this study on the logical assumption that comparative investigations of these tissues would provide a greater understanding of the metabolic variance associated with both normal and abnormal cellular proliferation. The following is a brief review of those features of the metabolism of foetal, regenerating and neoplastic liver tissues which relate to cell growth, proliferation and the pentose phosphate pathway. It is shown that these 'growth' tissues share a number of common features and tend to resemble each other more closely than they resemble normal adult liver.

1.4.2 Glucose phosphorylation

In liver, the first rate-limiting step in glucose utilization is its phosphorylation to glucose 6-phosphate. Two enzymes for the phosphorylation of glucose are present in adult liver, a specific glucokinase (EC 2.7.1.2) and a non-specific hexokinase (EC 2.7.1.1.) with a much lower Michaelis constant ($K_m$) for glucose. The maximum catalytic capacity of glucokinase is 3 fold greater than that of hexokinase in adult liver (Knox, 1972). Glucokinase is absent from foetal rat, guinea-pig and lamb liver (Walker and Holland, 1965; Ballard and Oliver 1964 , 1965). In response to partial hepatectomy, the pattern of these two enzymes tends to be more similar to that of foetal than adult liver. Knox (1972) reported a 1.56 fold increase in the activity of hexokinase in regenerating liver and a decrease of glucokinase activity to 0.56 of the normal adult liver value. The levels of glucokinase are also depressed significantly in hepatomas and the activity of hexokinase rises relative to normal liver (Criss, 1971; Knox, 1972; Shapira, 1973). This alteration from the normal adult liver pattern in hepatomas correlates with tumour growth rate (Weber *et al.*, 1977) but is not transformation-linked since at least one transplantable Morris hepatoma, the 9618A, has nearly normal levels of these two enzymes (Farina *et al.*, 1974). In terms of metabolism it is considered that hexokinase acts on glucose at low concentration for cell maintenance, while glucokinase appears only in mature liver tissue to capture glucose at higher than normal blood glucose concentrations for storage as glycogen. Thus in 'growth' tissues, the change in enzyme profile suggests an

increased capacity to utilize glucose as a consequence of the greater potential for its phosphorylation *via* hexokinase.

### 1.4.3 Glycolysis and the tricarboxylic acid cycle

The rate of anaerobic glycolysis is similar in foetal and adult rat liver slices but under anaerobic conditions foetal liver slices produce more lactate than adult liver (Villee and Hagerman, 1958; Villee *et al.*, 1958). Individual enzymes of the tricarboxylic acid cycle and of the electron transport chain are of low activity in homogenates of rat liver at term although there is a sharp rise to adult levels in the first few days of life (Potter *et al.*, 1945; Dawkins, 1959). The utilization of glucose 6-phosphate *via* glycolysis in regenerating liver is complicated by compartmentation of this pathway. Siebert and co-workers reported that intranuclear glycolytic activity is significantly increased, becoming maximal at 12h post-hepatectomy, while cytoplasmic glycolysis is actually depressed (Siebert, 1960, 1961; Siebert *et al.*, 1961). The glycolytic rate in neoplastic hepatic tissues, particularly the Morris hepatomas is an area of some controversy in the literature. Warburg (1931), observed that, in the tumours he examined, respiration was low and glycolysis high relative to normal tissues. On the basis of these studies, Warburg proposed that the carcinogenic agent interferes with cell respiration and the cell either dies or adopts a fermentative mechanism to derive energy necessary for survival. Weinhouse (1956) disagreed with the basic concepts of Warburg as a critical biochemical characteristic of

neoplasia and Pitot (1963) pointed out a number of biochemical characteristics of the first of the Morris "minimal deviation" hepatomas and noted in particular that the Morris hepatoma 5123 (a subline of which has been used for these studies) exhibited no increased aerobic glycolysis. Burk *et al.* (1967) however, re-examined the glycolytic capacities of a number of Morris hepatomas (including the 5123 series) and argued strongly that all hepatomas show glucose fermentation values in excess of those in normal or host rat livers and noted a continuous, positive correlation between *in vivo* growth rate and *in vitro* anaerobic glycolysis. The results of the studies reported in this thesis support the conclusions of Burk *et al.* (1967) for the 5123 hepatoma (see Chapter 4). Thus it appears that a common feature of hepatic 'growth' tissues is an increased glycolytic capacity, although the case for regenerating liver is not substantial in terms of total cell metabolism.

### 1.4.4 Gluconeogenesis and glycogen metabolism

The synthesis of glucose from precursors and storage of glucose as glycogen are key metabolic functions of adult liver and are central to its role in maintaining a constant blood glucose concentration. The synthesis of glucose is a capability which develops after birth (Greengard, 1971), is impaired in regenerating liver (Taketa *et al.*, 1976; Bonney *et al.*, 1973) and absent from most hepatomas (Knox, 1972; Weber *et al.*, 1977). Glycogen is rapidly lost following partial hepatectomy and the glycogen content of hepatomas is very low. Hepatic synthesis of glycogen in intact foetal liver is very rapid in the last stages of

gestation (Goldwater and Stetten, 1947) with glucose being the major precursor (Ballard and Oliver, 1964 ). These glycogen stores are rapidly lost immediately after birth in all mammalian species examined (Shelly, 1961). Thus, with the exception of foetal liver in the late stages of gestation, these two functions of adult liver, i.e. gluconeogenesis and glycogen storage, are not characteristic of hepatic 'growth' tissues.

### 1.4.5 Nucleic acid synthesis

Of the tissues under consideration, foetal liver from 14 days gestation to term is the fastest growing followed by regenerating liver (0-2 days after resection) and the hepatomas considerably slower (see Table 3.1). The rapid rate of cell division in these tissues implies increased synthesis of nucleic acids relative to "resting" adult liver. In regenerating liver, DNA synthesis exhibits an abrupt rise at around 15-18h following surgery. There is an active incorporation of labelled deoxyribotide precursor which reaches a peak at 22-26h and then diminishes somewhat but continues above basal level for some days (Hecht and Potter, 1956; Bucher, 1963). For this reason, all isotopic tracer studies reported in this thesis were performed on regenerating liver 24h following partial hepatectomy.

### 1.4.6 Lipid synthesis

The synthesis of lipid by growing hepatic tissues is of particular interest to this study since this biosynthetic process requires hydrogen in the form of NADPH. Large amounts of lipid, mostly triglyceride, appear in foetal

liver at term and disappear in the first few days of life (Freedman and Nemeth, 1961). Ballard and Hanson (1967) reported the rate of synthesis of non-saponifiable lipids (mostly cholesterol) from glucose to be 15 times greater in the liver of the 18 day rat foetus than in adult liver. There is also a dramatic accumulation of neutral lipid in parenchymal cells of the rat liver remnant following partial hepatectomy. The lipid that accumulates is predominately triglyceride and may increase 4 fold 20h after partial hepatectomy (Glende and Morgan, 1968; Fex, 1970). The transient fatty nature of the regenerating liver is associated with increased incorporation of fatty acids from plasma into liver triglycerides (Fex and Olivecrona, 1968b), an increased mobilization of free fatty acids from adipose tissue, and increased *de novo* synthesis of fatty acids in liver (Gove and Hems, 1978). The rate of lipid synthesis *in vitro* may vary considerably between tumours and may be more or less than, or the same as that by normal liver. Sabine *et al*. (1968) observed no relationship between tumour growth rate and the rate of fatty acid synthesis *in vitro*. However, Siperstein (1970) and McGarry and Foster (1969) presented data which suggests some correlation between cholesterol synthesis and the degree of differentiation of hepatomas. An interesting and consistent feature of lipid synthesis by hepatomas is the failure of these tissue to respond to normal dietary regulation such as fasting or feeding a high cholesterol diet. Thus lipid synthesis in hepatomas, although it may not be elevated, is unregulated. The increased rates of lipid synthesis in growing hepatic tissues, or in the case of the hepatomas the unregulated

rates, are significant in relation to the flux of glucose through the pentose phosphate pathway, since this flux is accompanied by NADPH production. This mechanism for NADPH production may be especially important in foetal liver since "malic" enzyme is not present in this tissue (Ballard and Hanson, 1967).

### 1.4.7 The pentose phosphate pathway

It is not possible to reach a conclusion about the role of the pentose phosphate pathway in 'growing' hepatic tissues based on information in the literature. Burch *et al*. (1963) reported the maximum catalytic capacity of glucose 6-phosphate dehydrogenase to be greater in foetal and new-born rat liver than adult, however Knox (1972) reports values averaging only 70% of adult liver for this tissue. Weber (1973) reported no alteration in the activity of glucose 6-phosphate dehydrogenase in regenerating liver, whereas Taketa *et al*. (1976) reported a 1.5 fold increase in the activity of this enzyme following partial hepatectomy and acute $CCl_4$ intoxication. The activity of glucose 6-phosphate dehydrogenase is dramatically increased in all hepatomas (10 fold) (Weber *et al*., 1977). Measurements of the F-type pentose cycle using the methods described above (Section 1.3) provide little useful information regarding the role of the pentose pathway in hepatic tissues. Crockett and Leslie (1963) reported values between 10-20% based on $^{14}CO_2$ yields as described in Section 1.3.2 in human foetal liver cells in culture. Katz and Rognstad (1967) analyzed the data of Horecker *et al*. (1958) and concluded the contribution of the pentose cycle is approximately 20% in

regenerating liver. It is emphasized that neither of these measurements can be compared to normal liver since no accurate estimate of the L-type cycle is known in this tissue and further the estimates for these two 'growth' tissues may be in considerable error if it is shown that the L-type cycle operates in these tissues.

### 1.4.9 Conclusions

Apart from nucleic acid synthesis, it is not clear if any of the metabolic variances discussed above are directly related to cell growth and proliferation. Changes such as increased lipid synthesis and the activity of glucose 6-phosphate dehydrogenase are also seen in non-proliferating hepatic tissues as a result of alterations in diet and other factors, and certain characteristics discussed above for foetal liver can easily be explained in terms of such changes in the environment of these cells. Nevertheless, the similarities discussed above indicate that a number of deviations from normal adult liver metabolism occur in these 'growth' tissues and these must be considered when interpreting data with respect to glucose metabolism and the pentose phosphate pathway. It is considered that a trend away from the homeostatic functions of adult liver and towards a more autonomous metabolism constitutes a reasonable generalization of the metabolism occurring in these 'growth' tissues.

## 1.5 Host Liver

That tumour-bearing results in alterations in the enzmatic profile of distant "non-involved" organs such as

host liver was first reported by Greenstein (1947) and has been confirmed by Suda *et al.* (1966); Wu and Homburger (1969); Herzfeld and Greengard (1972) and Knox (1972). It is of interest to this study that, in general, the alterations in enzymatic profile of host liver diverges towards that of the immature liver and the well-differentiated hepatomas. These alterations include decreases in glucokinase and increases in hexokinase activity (Herzfeld and Greengard, 1972). Very little is known regarding the effect of changes in enzymic profile induced by tumour-bearing, particularly as it effects the tumour-host relationship and metabolism, and nothing is known regarding alterations in the pentose phosphate cycle in host liver. It is for this reason that host liver tissue was included in all of the early studies described in this thesis.

## 1.6 Retrodifferentiation in liver regeneration and neoplasia

As discussed above (Section 1.4.9), the general picture of glucose metabolism in hepatomas and to a lesser extent in regenerating liver resembles that of foetal liver. The strategy of growing hepatic tissues appears to be that of assuming the enzymatic and metabolic profile of less differentiated, immature tissue. This phenomena is not limited to glucose metabolism. The evidence which has accumulated over the past 20 years of the expression of embryonic and foetal gene products is now so impressive as to preclude any doubt that it is a general characteristic of cancer. These embryonic gene products include ectopic hormone production, isoenzyme forms and antigens and

related immunological phenomena. The production of oncofoetal proteins by malignant cells is an area of considerable interest and a number of reviews of the subject are available (Coggin and Anderson, 1974; Gurchot, 1975; Fishman, 1976; Fishman and Singer, 1976; Uriel, 1976; Longenecker and Williams, 1977). It is of interest to the present study that few, if any, of the now known early developmental proteins associated with tumours are specific for neoplasia since they are often associated with cellular regeneration and metaplasia. For example, liver regeneration in rats and mice gives rise to morphological alterations coincidental with those of foetal or neonatal hepatocytes (Bresnick, 1971). These structural changes have also been observed in hyperplastic nodules of rat hepatocytes appearing in the early, preneoplastic phase of liver carcinogenesis by diethylnitrosamine (Bruni, 1973). The regeneration period is also characterized by increased synthesis of foetal-specific proteins seen as a transitory shift in isoenzyme pattern (Uriel, 1976). These correlations between regenerating and neoplastic liver have led to the proposal that early developmental gene expression is a consequence of the general adaptive process of retrodifferentiation (Uriel, 1976). Retrodifferentiation is defined as the stepwise nucleocytoplasmic reversion of mature elements toward a more juvenile state. The period of retrodifferentiation associated with regeneration is normally counterbalanced by a process of reontogeny which restores the terminal phenotype. The effects of cell oncogenic agents may be seen as blocking the reontogeny process resulting in an unbalanced retrodifferentiated cell which is

malignant. It is this phenomenon of blocked reontogeny which clearly differentiates the effects of carcinogenic agents from the toxic effects of non-carcinogens. The process of retrodifferentiation is a convenient mechanism to explain the re-expression of early developmental gene products in neoplastic and regenerating hepatic tissue. In terms of metabolism, this proposition predicts a predominance of autonomous foetal-like metabolic pathways over adult-type. The inability of hepatomas to store glycogen and their high glycolytic capacities may be seen as examples of the effects of retrodifferentiation on the metabolism of these tissues since these effects are a consequence of changes in isoenzyme composition and/or loss of adult-type enzymes. If the pentose pathway flux is critical to cell growth and proliferation, it may be considered that both the flux through, and the mechanism of, the pathway might be different in foetal, regenerating and neoplastic liver from that of the fully differentiated adult liver. The question then arises, is the L-type pentose pathway a liver-specific, adult form of the pathway and does a foetal form (i.e. the F-type or other) exist? These considerations indicated a requirement to examine the pentose pathway from a qualitative as well as a quantitative view.

## 1.7 The aim of this study

The major portion of the work described in this thesis was originally initiated as a consequence of the results and observations of J.F. Williams, M.G. Clark, and P.F. Blackmore that an alternate reaction scheme for the pentose phosphate pathway existed in liver (Williams *et al.*,

1978ab). It was considered that the elucidation of this reaction scheme offered a unique opportunity for reinvestigating the contribution of the pentose cycle to the metabolism of glucose by liver tissues.

This study has included investigations of foetal, regenerating and neoplastic hepatic tissue since it is generally held that the primary physiological role of the pentose phosphate pathway is the provision of metabolic intermediates required for cell growth and division. It is the main aim of the present study to investigate the participation of the pentose phosphate pathway in normal and rapidly proliferating hepatic tissues in an attempt to determine any quantitative and/or qualitative relationships between pathway flux and/or reaction mechanism and cellular proliferation.

# CHAPTER 2

## EXPERIMENTAL

### 2.1 Enzymes and chemicals

Unless otherwise stated, all enzymes, scintillants, substrates, buffers and coenzymes were obtained from either Boehringer Corp. (London) Ltd., Calbiochem (Australia) Pty. Ltd., Carlingford, N.S.W. or from the Sigma Chemical Co., St. Louis, Mo., U.S.A. [1-$^{14}$C], [2-$^{14}$C], [6-$^{14}$C] and [U-$^{14}$C] glucose, [2-$^{14}$C] and [U-$^{14}$C] glycerol were obtained from the Radiochemical Centre, Amersham, Bucks, England. Inorganic and organic solvents were analytical grade (E. Merck, Darmstadt; the British Drug House Ltd. or May and Baker Ltd., England). Glass distilled water was used to make all solutions.

All gases were supplied by Commonwealth Industrial Gases Pty. Ltd., Sydney, Australia.

Bio-Rad and Amberlite ion-exchange resins were obtained from Bio-Rad Laboratories, Richmond, California, U.S.A. and Rohm and Hass Co., Philadelphia, U.S.A. respectively.

### 2.2 Animals and diet

The Morris hepatoma 5123C was obtained from the Waite Agricultural Research Institute, Adelaide, South Australia, and the Morris hepatoma 7777 was obtained from Professor Harold P. Morris, Department of Biochemistry, School of Medicine, Howard University, Washington D.C. Inbred Buffalo rat stocks were obtained from Professor

Gerhard Schreiber, Russell Grimwa de School of Biochemistry, University of Melbourne, Victoria.

The Buffalo rats were maintained closely inbred by sibling matings in our animal house. Morris hepatomas 5123C and 7777 were maintained by serial transplantation in male inbred Buffalo rats. The transplantation was achieved by intramuscular injection of a uniform piece of tumour tissue (approx. 100 mg) using a 12 guage trochar, into the hind flanks of 8 to 10 week old animals.

Animals were bred and housed (4 to a cage) in a temperature controlled animal house which was maintained at 22°. The animal house was fitted with automatic lighting equipment which was programmed for a 12h light/dark cycle which commenced at 8 am (light cycle). All animals used in experiments were killed between 9 am and 10 am to minimise diurnal variation unless otherwise specified.

## 2.3 Enzyme preparations from tissues

Enzyme preparations of the following tissues were made, foetal, regenerating, host and adult liver and hepatomas 7777 and 5123C. The preparations consisted of the 105,000 supernatant fraction of the homogenized tissue. The preparations were made as follows: The animals were killed by cervical dislocation and the entire liver or tumour tissue removed, sliced into small pieces (approx. 0.2g), rinsed with 0.05M-Tris-HCl, pH 7.4, buffer to remove blood, weighed, and homogenized in 20 ml of 0.05 M-Tris-HCl buffer, pH 7.4, in a glass Potter-Elvehjem homogenizer using a motor-driven Teflon pestle. Special care was taken to remove all muscle, connective and necrotic tissue when excising the hepatomas. The entire homogenate was then diluted to a concentration of 1:8,

tissue:buffer (w/v) with 0.05 M Tris-HCl, pH 7.4, and centrifuged at 10,000 g for 10 min in a refrigerated Sorval centrifuge fitted with a SS-34 rotor. The resulting supernatant fluid was centrifuged at 105,000 g in a Beckman ultracentrifuge (model L-2) at 0-4$^{o}$ for 1h using a L-50 rotor. The supernatant fluid was strained through glass wool to remove lipid residues and the clear fluid was then dialyzed for at least 12h against two changes (1 litre each) of 0.05 M-Tris-HCl buffer, pH 7.4, at 0-4$^{o}$. The dialysis tubing (Visking Co., Chicago, U.S.A.) was first treated to remove plasticizing substances (Garland *et al.*, 1964).

## 2.4 Protein estimation

Protein concentrations were determined in enzyme preparations by the method of Lowry *et al.* (1951) using albumin (bovine), fraction V as standard.

## 2.5 Spectrophotometric measurements

Enzymatic and substrate assays were carried out in one of the following automatic instruments: Carl Zeiss PMQ II spectrophotometer (Oberkochen/Wuertt, West Germany); Varian Techtron UV-VIS double beam spectrophotometer model 635 (Varian Techtron Pty. Ltd., Melbourne, Australia) or Beckman Acta spectrophotometer model MVI (Beckman Instruments, Inc., Fullerton Ca., U.S.A.). All instruments were fitted with thermostatic temperature control, chart recorder and multi-cell carriages.

The growth of *Leuconostoc mesenteroides* in liquid media was monitored by observing the turbidity using a

Klett Summerson colourimeter.

## 2.6 Enzyme activities

The enzymes catalyzing reactions of the pentose phosphate pathway described by Horecker *et al.* (1954) were assayed for maximum catalytic activity in the cytosolic extract of livers and hepatomas. Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were assayed by the method of Glock and McLean (1953). The catalytic activities of transaldolase, ribose phosphate isomerase and ribulose phosphate 3-epimerase were determined as described by Clark *et al.* (1972). Transketolase was determined by the method (b) of Clark *et al.* (1972) and aldolase by the method of Raijkuman *et al.* (1966).

## 2.7 Intermediate levels in extracts from fresh tissue and isolated cells

The levels of sugar phosphate intermediates were determined from $HClO_4$ extracts of "freeze clamped" tissues. The procedure used was essentially as described by Wollenberger *et al.* (1960). Rats were killed by cervical dislocation and the liver was rapidly removed and clamped in aluminium tongs precooled to $-170^{o}$ in liquid nitrogen. Acid extracts of this frozen tissue were made by the addition of 10ml of ice cold $HClO_4$ (0.6N) followed by homogenization using a Potter-Elvehjem tissue homogenizer fitted with a motor driven Teflon pestle. The denatured protein was removed by centrifugation and the supernatant fluid adjusted to pH 6.5 with KOH solution. After removal of the $KClO_4$ precipitate by centrifugation (10,000g for 10 min), the

final volume was measured and the supernatant fluid removed for analysis of the intermediates.

The levels of adenine nucleotides and glucose 6-phosphate and fructose 6-phosphate in acid extracts of isolated cells were determined as an indication of the metabolic viability of the cell preparations. The cells were incubated for 1 hour at 37$^{o}$ in Krebs-Ringer Bicarbonate buffer pH 7.4 containing 5mM glucose, 0.2% fatty acid free-BSA and 10.0mM HEPES at a concentration of approximately 100mg wet weight of cells per ml. A 10ml sample of cells was then centrifuged at approx. 50g for 5 min and the supernatant fluid removed by suction. Five ml of 0.6M $HClO_4$ was then added to the cell pellet and the cells vigorously resuspended. This suspension was allowed to stand on ice for 15 min prior to the removal of denatured protein by centrifugation (10,000g for 10 min). The supernatant fluid was adjusted to pH 6.5 with KOH solution and the $KClO_4$ precipitate removed by centrifugation (10,000g for 10 min). The supernatant fluid was used directly for analysis of intermediates as described (Section 2.8).

## 2.8 Enzymatic analysis of sugar phosphates

Measurements of intermediates from "freeze clamped" tissues, extracts of isolated cell preparations and extracts of enzyme incubations with ribose 5-phosphate were made as follows: adenosine 5'-triphosphate (Lamprecht and Trautschold, 1974); adenosine 5'-diphosphate and adenosine 5'-monophosphate (Jarowek *et al.*, 1974); lactate (Gawehn) and Bergmeyer, 1974); pyruvate (Czok and Lamprecht, 1974); malate (Gutmann and Wahlefeld, 1974); glyceraldehyde

3-phosphate, xylulose 5-phosphate, ribulose 5-phosphate and ribose 5-phosphate (Gumaa and McLean, 1969); sedohepulose 7-phosphate (Racker, 1974); glucose 6-phosphate and fructose 6-phosphate (Lang and Michal, 1974); and 6-phosphogluconate (Haid, 1974).

The Atkinson "energy charge" ratio (Atkinson and Walton, 1967) was used in order to establish that the physiological integrity of each tissue "freeze clamp" preparation was maintained and was calculated by the following empirical equation.

$$\text{"ENERGY CHARGE"} = \frac{1}{2} \times \frac{(\ 2 \times [ATP] + [ADP]\ )}{(\ [ATP] + [ADP] + [AMP]\ )}$$

The cytoplasmic pyridine nucleotide ratios, $[NAD^+]/[NADH]$ and $[NADP^+]/[NADPH]$, were measured using the lactate dehydrogenase system and the malate dehydrogenase system respectively (Krebs and Veech 1968, Veech *et al.*, 1970). The ratios are determined from the relationships below:

$$\frac{[NAD^+]}{[NADH]} = \frac{[\text{pyruvate}]}{[\text{lactate}]} \times \frac{1}{1.11 \times 10^{-4}}$$

$$\frac{[NADP^+]}{[NADPH]} = \frac{[\text{pyruvate}]}{[\text{malate}]} \times \frac{[CO_2]}{3.44 \times 10^{-2}}$$

The $CO_2$ concentration was taken to be $1.16 \times 10^{-3}$ M. This is the level cited by Krebs and Veech (1968) for normal rat liver and it was assumed to be the same in all tissues studied.

## 2.9 Ribose 5-phosphate utilization and carbon balance studies

The rates of ribose 5-phosphate utilization, triose phosphate formation and hexose 6-phosphate formation during an incubation of the cytosolic extract from each tissue with 5mM ribose 5-phosphate were determined by assaying $HClO_4$ extracts of aliquots of the incubation mixture for the following sugar phosphates: ribose 5-phosphate, ribulose 5-phosphate, xylulose 5-phosphate, sedoheptulose 7-phosphate, glyceraldehyde 3-phosphate and dihydroxy-acetone phosphate, fructose 1,6 bisphosphate, glucose 6-phosphate and fructose 6-phosphate. The incubation mixture (10ml) contained 50 µmole ribose 5-phosphate, approx. 25 mg (protein) of the dialyzed 105,000 g supernatant solution and glycyl-glycine buffer (pH 7.4) to a final concentration of 0.1M. The incubation was at 25° for 2 hrs. A one ml aliquot of this mixture was added to an equal volume of 0.6M $HClO_4$ on ice at 0,2,6,15,30,60,90 and 120 min. The denatured protein was removed by centrifugation (10,000g for 10 min) and the supernatant solution adjusted to pH 6.5 with KOH solution. After removal of the $KClO_4$ precipitate by centrifugation (10,000g for 10 min), the final volume was adjusted to 2.5ml and the samples were removed for the analysis of the intermediates by the methods described above (Section 2.8). The carbon balance is expressed as µCatoms per mg of protein for the purpose of comparing the ability of the tissue extracts to catabolize ribose 5-phosphate.

## 2.10 Synthesis of D-*glycero*-D-*ido*-octulose 1,8-bisphosphate

D-*glycero*-D-*ido*-Octulose 1,8-bisphosphate was formed by the aldolase-catalysed reaction of equation (2.1).

D-Arabinose 5-phosphate + dihydroxyacetone phosphate $\rightleftharpoons$ D-*glycero*-D-*ido*-octulose 1,8-bisphosphate (2.1)

The reaction mixture contained in a total volume of 30 ml : 2.5 mmol of D-arabinose 5-phosphate (12.5-fold excess); 200 µmol of dihydroxyacetone phosphate; 30 units of rabbit muscle aldolase and 2.0 mmol of triethanolamine/HCl buffer, pH 7.6. The reaction was allowed to proceed at 30° for 40-45 hours. The formation of octulose bisphosphate was monitored using the cysteine-sulphuric acid method (Section 2.16). After 40 hours, the absorbance at 451 nm indicated a 75% conversion of the reactants to the octulose product. The reaction was terminated by the addition of perchloric acid to a final concentration of 0.6 M. The protein precipitate was removed by centrifugation (20,000g for 10 min) in a Sorvall refrigerated centrifuge. The protein-free supernatant solution was adjusted to pH 6.8 with KOH and allowed to stand on ice for 30-60 mins. Potassium perchlorate was removed by centrifugation as above and the supernatant fluid was pumped onto a column (1.5 x 30 cm) of Bio Rad AG 1-x8, 200-400 mesh, ion-exchange resin (formate form). The *ido*-octulose bisphosphate and unreacted arabinose 5-phosphate were separated by elution as described in Section 2.15. The fractions were monitored using the orcinol and

cysteine-sulphuric acid (Section 2.16) methods to detect the presence of pentose phosphate and *ido*-octulose bisphosphate respectively. Those fractions containing *ido*-octulose bisphosphate were pooled. The column eluate was continuously extracted with diethyl ether for 24 hours to remove formic acid. The pH of the ether-extracted material was adjusted to 6.8 with NaOH, and the solution concentrated by rotary film evaporation. The final yield of D-*glycero*-D-*ido*-octulose 1,8-bisphosphate was 60% based on the quantity of dihydroxyacetone phosphate originally in the incubation mixture.

The identity and purity of the product was confirmed using mass spectroscopy (courtesy of Dr. John MacLeod, The Research School of Chemistry, A.N.U.).

## 2.11 Hexose 6-phosphate formation from D-*glycero*-D-*ido*-octulose 1,8-bisphosphate

The rate of hexose 6-phosphate formation from the substrates D-*glycero*-D-*ido*-octulose 1,8-bisphosphate and *altro*-heptulose 7-phosphate was determined as follows. The reaction mixture contained 14 µmol of KCl; 18 µmol of triethanolamine/HCl buffer, pH 7.6; 2.2 µmol of the *ido*-isomer of octulose 1,8-bisphosphate; 2.2 µmol *altro*-heptulose 7-phosphate; 0.1 mg of thiamine pyrophosphate; rat enzyme preparation (1.25 mg of protein) and water to a volume of 0.44 ml. The reaction was initiated by the addition of enzyme and 60 µl samples were removed at time zero and at 5, 10, 15, 20, 30 and 40 min. The samples were added directly to 50 µl of 0.6 M perchloric acid and the denatured protein removed by centrifugation. The supernatant fluid

was then neutralized (pH 6.5-7.0) with saturated potassium bicarbonate, the potassium perchlorate precipitate removed by centrifugation and 100 µl of the supernatant solution was taken for the analysis of hexose 6-phosphate. Glucose 6-phosphate and fructose 6-phosphate were determined enzymically as described by Lang and Michal (1974). For each experiment, control incubations were performed using only one substrate. Rates of hexose 6-phosphate formation were observed when *altro*-heptulose 7-phosphate was the sole substrate and this rate was subtracted from the rate observed for both substrates, to yield the net phosphotransferase-coupled reaction rate. Hexose 6-phosphate standards (10 nmol) were treated in an identical manner as the 60 µl time samples and the yield of hexose 6-phosphate in the final assay was 95%.

## 2.12 Preparation and incubation of isolated hepatocytes

Isolated hepatocytes were prepared by perfusion of the isolated rat liver or liver remnant with collagenase essentially as described by Berry (1974) except that hyaluronidase was omitted. Partial hepatectomy was performed as described by Higgins and Anderson (1931) and isolated cells were prepared 24 hrs after surgery. All cell preparations were performed between 9.00 a.m. and 10.00 a.m. to avoid any complications arising from diurnal rhythm. The cell preparations were judged to be greater than 90% viable by trypan blue exclusion.

The cells were incubated in Krebs-Ringer Bicarbonate buffer pH 7.4 containing 5mM glucose, 0.2% fatty acid free-BSA and 10.0 mM HEPES at a concentration of approx.

100 mg wet weight of cells per ml. The cell suspensions were gassed with $O_2$:$CO_2$; 95:5, prior to incubation. Experiments in which the incorporation of $^{14}C$-isotope from [1-$^{14}C$] and [6-$^{14}C$] glucose into $^{14}CO_2$ was measured were carried out in glass scintillation vials with a centre well containing 0.5ml of 2N NaOH. The vials were sealed with rubber stoppers (Suba seals). The volume of cell suspension in these incubations was 3ml, to which 0.2 µCi of $^{14}C$-labelled glucose was added. The incubations were from 15 min to 2h at 37°. Experiments in which $^{14}C$-isotope incorporation into lipid, DNA, RNA and protein were measured contained 5ml of the cell suspension and 2.5 µCi of either [1-$^{14}C$] or [6-$^{14}C$] glucose. These experiments were for 2 hrs at 37°C.

The incorporation of $^{14}C$ into lipid, RNA, DNA and protein was measured by fractionation of the cell suspension as follows. The incubation was terminated by the addition of 5.0 ml of 10% trichloroacetic acid and the solution allowed to stand on ice for 30 min. The suspension was then centrifuged (10,000 g -10 min) in a Sorval refrigerated centrifuge using the SS-34 rotor. The supernatant fluid was discarded and the pellet washed with a further 5.0 ml of 10% trichloroacetic acid. The pellet was resuspended in 5.0 ml of a solution of chloroform:methanol (2:1) by homogenization using a motor driver Teflon pestle and incubated at 37°C for 15 min. The solution was centrifuged (12,000 g -10 min) and the lipid extract retained. The pellet was resuspended in 3ml of the chloroform:methanol solution and the extraction procedure repeated. The combined lipid extracts were analyzed for radioactivity by

counting 0.1ml in 10ml of butyl-PBD scintillation fluid using a Beckman scintillation counter (model LS-100). The pellet was dried *in vacuo* and resuspended in 5.0ml of 0.3M KOH and incubated at 60°C for 3 hours to hydrolyze RNA. The incubation was terminated by the addition of 3.0ml of ice cold 1.2M $HClO_4$ and allowed to stand on ice 15 min prior to centrifugation (12,000 g -10 min). The supernatant was removed and 0.1ml of this RNA fraction counted in 10ml of butyl-PBD scintillant as above. The pellet was resuspended in 5ml of 0.6M $HClO_4$ and incubated at 70°C for 1 hour to hydrolyze DNA. The incubation was terminated by cooling on ice for 15 min and centrifugation (12,000 g-10 min). The supernatant fluid was retained and the pellet resuspended in 3.0ml of 0.6N $HClO_4$ and centrifuged as above. The supernatant fractions were combined and a 0.1ml sample analyzed for radioactivity as above. The pellet, containing denatured protein, was solubilized in 3ml of Soluene 350 (Packard Instrument Company Inc., Downers Grove, Ill., USA) by incubating overnight at 60°C. A 0.1ml aliquot of the solubilized protein was then counted in 10ml of scintillation fluid as above. All measurements of radioactivity were corrected for quenching (Section 2.14). The incubations from which glucose 6-phosphate was isolated for degradation following the metabolism of [2-$^{14}$C], [5-$^{14}$C] or [4,5,6-$^{14}$C] glucose contained 10ml of cell suspension and 10μCi of $^{14}$C labelled glucose. The recovery of glucose 6-phosphate, its dephosphorylation and degradation, and the synthesis of [5-$^{14}$] and [4,5,6-$^{14}$C] glucose are described elsewhere (Sections 2.17 and 2.19).

## 2.13 Growth of the 5123tc hepatoma in culture

The 5123tc hepatoma line was obtained from Dr. T.R. Bradley, Peter McCallum Cancer Institute, Melbourne, Australia. The cells were grown as a monolayer in 2 litre roller bottles containing 200ml of growth medium. The medium consisted of α modification of Eagles medium (Flow Laboratories Ltd., Annandale, N.S.W., Australia) containing foetal calf serum at a final concentration of 10% (v/v), penicillin (0.06 g/l) and streptomycin (0.1 g/l). The cells were harvested and subcultured weekly by seeding at $10^7$ cells per bottle. The culture medium was changed every two days. Cells were released from the glass with 0.05% trypsin, and the cell density determined by counting the cells with the aid of a haemocytometer (Assistent-Germany). Cell stocks were stored in liquid nitrogen. The harvested cells were suspended at a concentration of approx. 100 mg wet weight of cells per ml in growth media prior to use in $^{14}C$-isotope studies. All other incubation conditions were as described above for hepatocytes (Section 2.12).

## 2.14 Radioactivity measurements

Radioactivity measurements were made using either a Beckman LS-350, Beckman LS-100 or a Packard Tri-carb (model 2002) scintillation counter. The scintillant contained 12g of 2(4'-t-butylphenyl)-5-(4"-biphenyl)-1,3,4-oxadiazole, 1200ml of toluene and 800ml of methoxyethanol. All counting was corrected for quenching by the channels ratio method (Bruno and Christian, 1961).

Radioactive sugars and sugar phosphates were located on paper chromatograms by scanning for radioactivity

using a Packard Model 7201 Radiochromatogram Scanner.

## 2.15 Separation of sugar phosphates by ion-exchange chromatography

Sugar phosphates were separated from one another by an ion-exchange chromatography using Dowex-1(x8, 200-400 mesh) analytical grade resin in the formate form. The samples were loaded onto a column (1.5 x 30 cm) followed by 50ml of water. Elution was achieved using a linear gradient containing 500ml of 2M HCOOH in a mixing bottle and 4M HCOOH in the reservoir. Fractions (8-10ml) were collected at a constant flow rate of 1.0ml/min in an Isco Golden Retriever, Model 820 fraction collector (Instrument Specialties Co. Inc., Lincoln, Nebraska, U.S.A.). The flow was maintained with an LKB Re Cy Chrom Pump, type 4912A (LKB-Produkter AB, Stockholm, Sweden).

## 2.16 Colorimetric methods for estimating sugar phosphates

The main application of the colorimetric methods employed was in determining the location of sugar phosphates in fractions following ion-exchange chromatography. Total aldo pentose 5-phosphate was estimated using the modified orcinol melthod of Blackmore and Williams (1974). The location of glucose 6-phosphate and fructose 6-phosphate in fractions following ion-exchange chromatoggraphy was determined using the anthrone method of Graham and Smydzuk (1965).

The octulose phosphates were characterized and measured using the unique spectra of their reaction

products formed by the following modification of the cysteine-sulphuric acid method of Bartlett and Bucolo (1968). In a 15cm x 1.5cm test tube 0.5ml of sample (containing 5-100 nmol of octulose phosphate) was mixed with 1.5ml of 18M sulphuric acid and immediately placed in an ice bath for 3 min. The tube was capped with a glass marble and heated in a vigorously boiling water bath (3 min) then cooled on ice (2 min). To this mixture was added 33 µl of freshly prepared 3% (w/v) cysteine-HCl solution and this mixture heated at 100$^{o}$ for 3 min. The reaction mixture was then cooled in an ice bath for 15 min and the spectra recorded between 350 and 500 nm (Paoletti *et al.*, 1979a).

2.17 Synthesis of [5-$^{14}$C] and [4,5,6-$^{14}$] glucose

[5-$^{14}$C] glucose was prepared from [2-$^{14}$C] glycerol and [4,5,6-$^{14}$C] glucose from [U-$^{14}$C] glycerol using a modification of the method described by Ljungdahl *et al.* (1961). The reaction mixture (5.0ml) contained: 100µmole ATP; 200µmole $NAD^{+}$; 150µmole fructose 6-phosphate; 1mg glycerokinase (EC 2.7.1.30; Boehringer Mannheim Corp.; from Candida mycoderma; 85U/mg); 1mg glycerol 3-phosphate dehydrogenase (EC 1.1.1.8; Boehringer Mannheim Corp., from rabbit muscle; 40U/mg); 100µg triose-phosphate isomerase (EC 5.3.1.1; Boehringer Mannheim Corp.; from rabbit muscle; 5000 U/mg); 1.3 mg transaldolase (EC 2.2.1.2; Boehringer Mannheim Corp.; from yeast; 15U/mg); 160µg lactate dehydrogenase (EC 1.1.1.27; Boehringer Mannheim Corp.; from rabbit muscle; 550 U/mg); 200µmole sodium pyruvate and 250µCi of [2-$^{14}$C] or [U-$^{14}$C] glycerol

(25mCi/mmole). All substrates, co-factors and enzymes were made up in 100mM Tris/HCl, 20mM $MgCl_2$ pH 7.8. The reaction mixture was incubated for 2 hrs. at 25° before the addition of 50U of phosphoglucose isomerase (EC 5.3.1.9; Boehringer Mannheim Corp.; from yeast; 350U/mg). The incubation was continued for one further hour and was then terminated by the addition of 5.0ml of 0.6M $HClO_4$. The mixture was allowed to stand in ice for 30 min. to complete the precipitation of protein. The protein was removed by centrifugation at 12,000g for 10 min. in a Sorval refrigerated centrifuge using the SS-34 rotor. The supernatant fluid was adjusted to pH 6.5 with KOH solution and allowed to stand at 0° for 30 min. The $KClO_4$ precipitate was removed by centrifugation as above and the reaction mixture placed on a column (1.5 x 30cm) of Dowex-1 (x8, 200-400mesh, formate form) analytical grade resin followed by 50ml of water. The column was eluted with a linear gradient as described in Section 2.15. Fractions (10ml) were collected and the hexose 6-phosphate peak identified using the anthrone reagent (Section 2.16) and the appropriate fractions were collected (total volume 6 x 10ml). The solution of $^{14}C$-labelled hexose 6-phosphates was concentrated under reduced pressure at 45° using a Buchi rotary evaporator (Buchi Rotovapor R). The last traces of formic acid were removed by adding 100 ml of water and evaporating the solution under reduced pressure. This process was repeated four times.

The glucose 6-phosphate was separated from fructose 6-phosphate by descending paper chromatography using the GW3 solvent of Wood (1968). The glucose 6-phosphate was recovered from the paper by elution with water and concentrated to 5.0ml under reduced pressure using a Buchi rotary evaporator.

The glucose 6-phosphate solution was made up to a volume of 10ml in a solution which was 100mM Tris/HCl, 10mM $MgCl_2$ pH 10.4 and dephosphorylated by the addition of 20mg of alkaline phosphatase (EC 3.1.3.1; Sigma Chemicals; from calf intestine; 2U/mg). The dephosphorylation incubation was maintained at 37° for 3 hrs and was terminated by the addition of 10ml of 0.6M $HClO_4$ and the precipitated protein removed by centrifugation as above. The supernatant solution was de-ionized by passing through tandem columns (1.2 x 20cm) of Dowex 50 ($H^+$ form) and Dowex 1 (x8, acetate form). The columns were washed with 100ml of water and the appropriate fractions were pooled and evaporated to near dryness *in vacuo* at 40° in a rotary film evaporator. The $^{14}C$-labelled glucose solution was evaporated to near dryness 4 times following each of 100ml additions of distilled water to remove acetic acid. The solution was then concentrated to a final volume of 2.0ml. Enzymic analysis of the glucose solution indicated that it was free from fructose. The specific radioactivity of the glucose was approximately 1.7mCi/mmole and the yield was 60μCi.

## 2.18 Growth conditions of *L. mesenteroides*

Freeze-dried cultures of *L. mesenteroides* strain 39 were obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland, U.K. (N.C.I.B. catalogue no. 8699) and were grown at 30° on the media described by De Man *et al.* (1960). For complete and rapid degradation of glucose it was mandatory that the culture was harvested at the "vigorously gassing" stage of growth (Sakami, 1955) which was towards the end of the exponential growth phase.

Measurements were made in a 250ml Erlenmeyer flask fitted with a 13mm diameter glass side arm which fitted into the cell housing Klett Summerson colourimeter (Section 2.5) the instrument was zeroed against sterile media contained in a similar flask. Subcultures, with 2% (v/v) inoculum were made from cultures having a cell density between 215-240 Klett units and harvesting of the final culture was carried out at this same cell density. The time between inoculation and subculture or harvest was 7-9h, the generation time being shorter in the larger cultures, probably due to the more favourable conditions for growth of microaerophils.

Cultures were harvested in a refrigerated Sorvall centrifuge (Ivan Sorvall Inc., Newtown, Connecticut, U.S.A.) using a GS-3 rotor. After washing twice in 1.0 M-$NaH_2PO_4$ buffer, pH 6.0, the total cell yield was suspended in the same buffer to give a concentration of 30% (w/v).

## 2.19 Degradation of $^{14}C$-glucose

### 2.19.1 Fermentation of glucose with *L. mesenteroides*

To variable amounts of glucose obtained from glucose 6-P (by dephosphorylation), unlabelled carrier glucose was added to give a final amount of 1 mmol and fermented at $30^{o}$ to $CO_2$, ethanol and lactic acid in 125ml Warburg flasks as described by Sakami (1955), the centre well containing 2ml of 2.5 M-NaOH to trap $CO_2$ (C-1 of glucose). A control containing no glucose was included in each batch of fermentations, and specific radioactivities were corrected for any dilution indicated by the yield of endogenous $CO_2$, this constituted 2-4% of that obtained from fermentation of 1 mmol of glucose. Approx. 5% of each

sample being fermented was retained so that a total combustion (Van Slyke and Folch, 1940) could be performed and hence the % recovery of label was able to be determined following the multi-staged degradation procedure. Each batch of fermentations was also accompanied by a duplicate [1,2,6-$^{14}$C] glucose standard of precisely known composition.

After 3.5h the fermentation was complete. The contents of the centre well was quantitatively transferred into 5ml of 0.4 M-$BaCl_2$. The contents of the main compartment were centrifuged (27 000 g for 10 min), ethanol and lactic acid were then isolated from the supernatant fluid (Sections 2.19.2; 2.19.3).

2.19.2 Isolation of ethanol and conversion to acetic acid

The supernatant solution obtained from the fermentation reaction mixture (approx. 35ml) was adjusted to pH 8.0 with 7.5 M-NaOH and ethanol was separated from lactic acid by short path distillation. Ethanol, which was completely removed when 25ml of distillate had been collected, was oxidized (12h) to acetic acid in a stoppered flask by the addition of 10ml of 4.5M-$H_2SO_4$ containing 17 mmol $CrO_3$. The acetic acid was recovered by steam distillation using a Markham still (Markham, 1942). The distillate, approx. 300ml, was collected directly into 20ml of 0.1M-NaOH. The solution was concentrated to approx. 10ml in a rotary film evaporator at 50$^{o}$, then transferred to reaction vessel B (100ml capacity) (Fig. 2.2) and evaporated to dryness.

2.19.3 Isolation of lactic acid

The residue from the ethanol distillation (Section

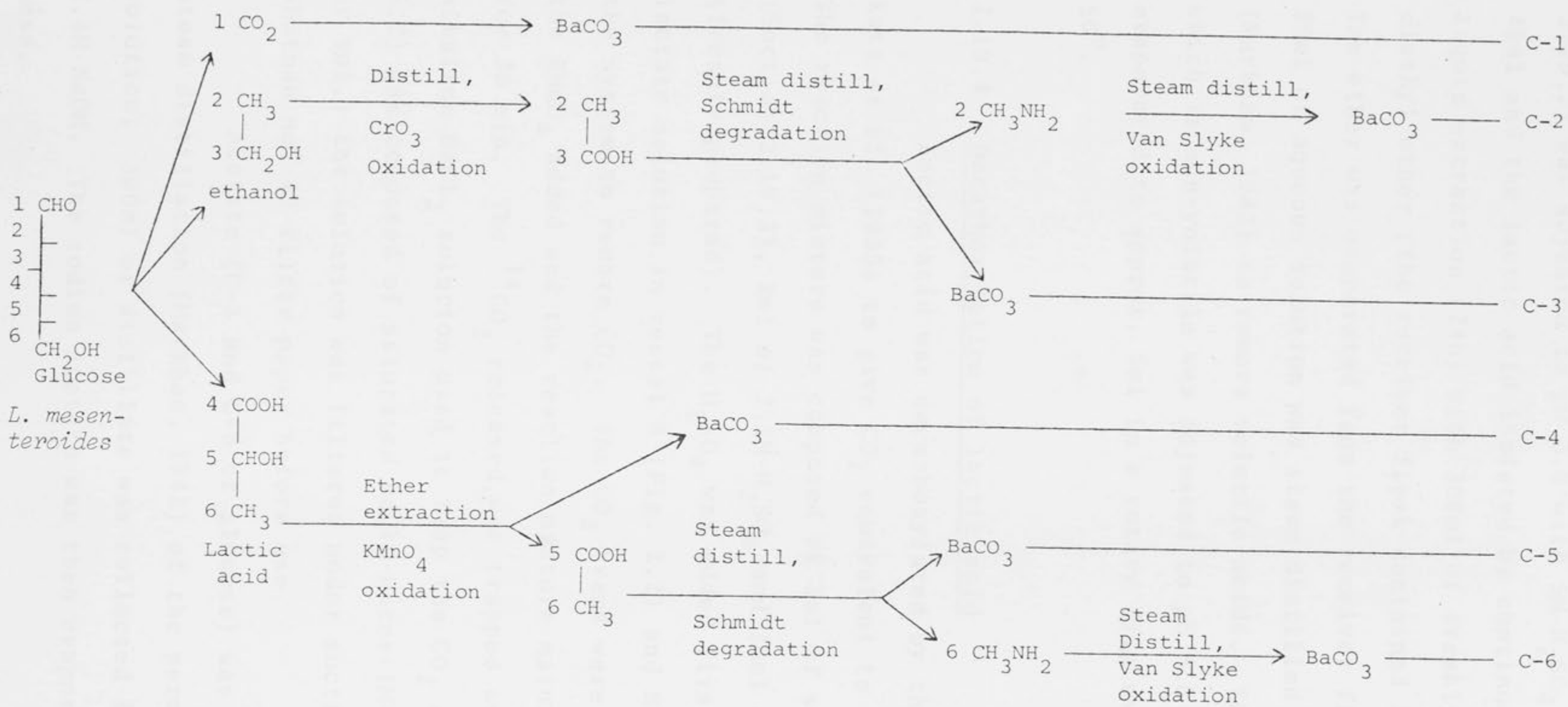



Fig. 2.1 Fate of glucose carbon atoms during degradation procedure described in Section 2.19

The numerals refer to the original position of the carbon atoms in the glucose molecule. Each carbon atom is trapped as $BaCO_3$ and counted as described in Section 2.20.

2.19.2) was adjusted to pH 3.0 with 5M-$H_2SO_4$, diluted to 40ml and the lactic acid isolated by continuous liquid-liquid extraction (24h) with 300ml of freshly distilled diethyl ether (the receiver flask contained 20ml of water). The ether was evaporated from the receiver flask and the 20ml of aqueous solution was steam distilled (1 vol.) (Markham, 1942) to remove volatile acids. The lactic acid which is non-volatile was adjusted to pH 8.0 with NaOH and evaporated to approx. 5ml in a rotary film evaporator at $50^{o}$.

2.19.4 Decarboxylation of lactic acid

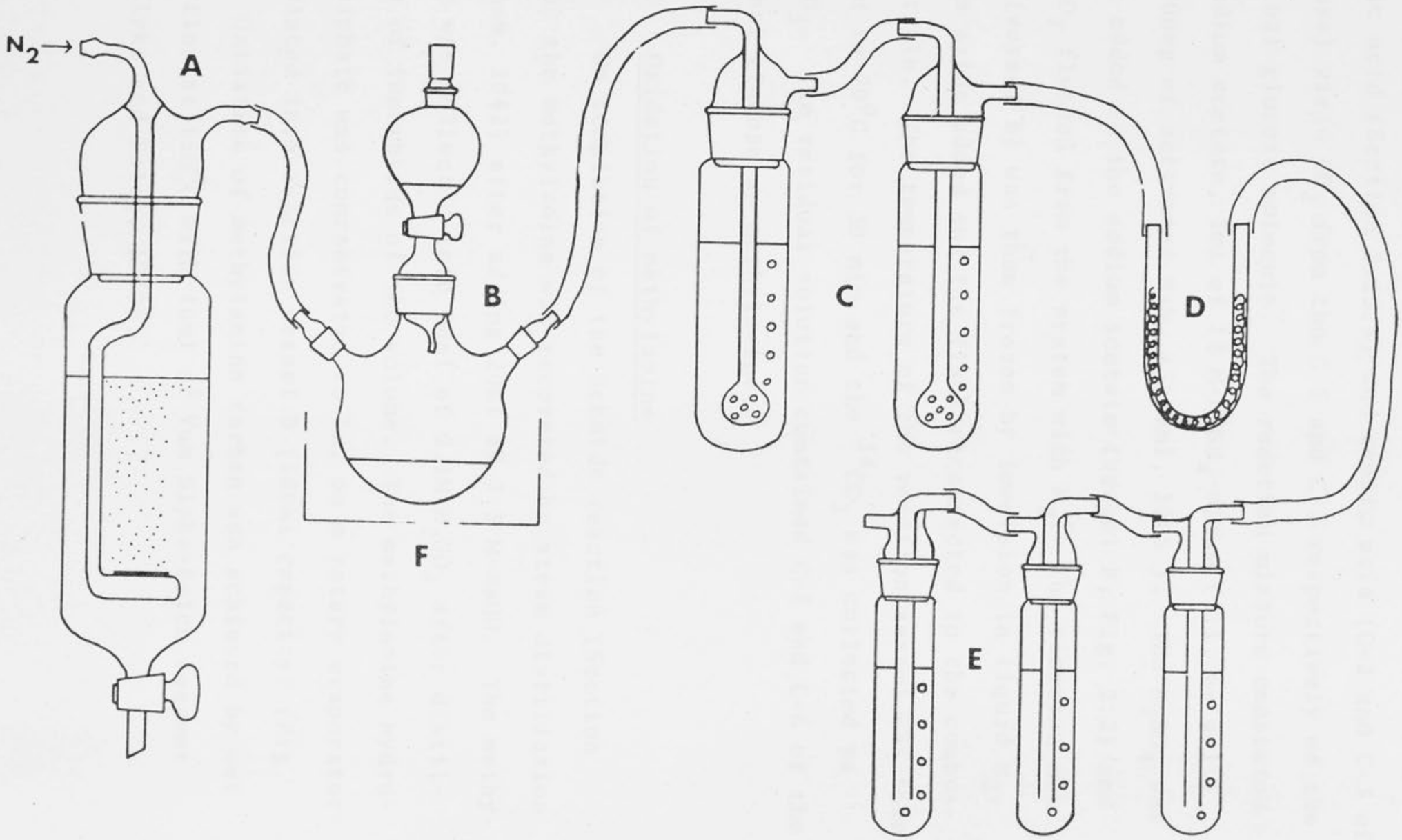
Lactic acid was decarboxylated by the method of Katz *et al.* (1955) to give $CO_2$ equivalent to C-4 of glucose. The reaction mixture was composed of 5ml of sodium lactate (Section 2.19.3), 2ml of 2.5M-$H_2SO_4$ and 20ml of 0.33M-$KMnO_4$ (freshly prepared). The $H_2SO_4$ was added first to the sodium lactate solution in vessel B (Fig. 2.2) and $N_2$ swept through the system to remove $CO_2$. The $CO_2$ traps were then connected, the $KMnO_4$ added and the reaction mixture maintained at $90^{o}C$ for 30 min. The $^{14}CO_2$ released was trapped as $Ba^{14}CO_3$. The alkaline $BaCl_2$ solution used to trap the $CO_2$ as $BaCO_3$ (Fig. 2.2) was composed of saturated $BaCl_2$-water-1M-NaOH (5:5:1, by vol.) the solution was filtered under suction through Whatman no. 42 filter paper before use.

Acetate (C-5 and C-6 of glucose) was recovered by steam distillation (Markham, 1942) of the permanganate solution; 300ml of distillate was collected into 20ml of 0.1M-NaOH. The sodium acetate was then evaporated to dryness.

Fig. 2.2 Combustion train for the degradation of lactic acid, acetic acid, methylamine, glucose and ribose

The combustion train was continuously flushed with $N_2$ which was first passed through a sparge (A) containing 1M-NaOH to remove any contaminant $CO_2$. $CO_2$ resulting from the oxidation of the above compounds in the reaction vessel (B) (100ml capacity for Schmidt degradation and 500ml capacity for $KMnO_4$ oxidation of lactic acid and Van Slyke combustion) was swept through two acid-permanganate scrubbers (C), [containing 4% (w/v) $KMnO_4$ in 0.25 M-$H_2SO_4$] to remove $SO_2$ and $SO_3$. The U-tube (D) was packed with glass wool to prevent the transfer of water vapour droplets. The $CO_2$ traps (E) contained alkaline $BaCl_2$. Temperature of the reaction vessel was maintained by either a thermo-regulated water-bath (F) or a Bunsen-burner in the case of the Van Slyke oxidation.

Fig. 2.2



2.19.5 Decarboxylation of acetic acid

Acetic acid was decarboxylated by the Schmidt oxidation (Phares, 1951). Acetate from the decarboxylation of lactic acid (Section 2.19.4) and acetic acid (C-2 and C-3 of glucose) yield $CO_2$ from the C-5 and C-3 respectively of the original glucose molecule. The reaction mixture consisted of sodium acetate, 2ml of 18 M-$H_2SO_4$-oleum (2:1, by vol.) and 200mg of activated $NaN_3$ (Sakami, 1955 ). The $H_2SO_4$ was first added to the sodium acetate (vessel B, Fig. 2.2) and the $CO_2$ flushed from the system with $N_2$. The reaction mixture (vessel B) was then frozen by immersion in liquid $N_2$, sodium azide added and the flask reconnected to the combustion train. The temperature of the reaction vessel was then raised to 70°C for 30 min and the $^{14}CO_2$ was collected as $Ba^{14}CO_3$. The residual solution contained C-2 and C-6 of the original glucose as methylamine.

2.19.6 Oxidation of methylamine

On completion of the Schmidt reaction (Section 2.19.5) the methylamine was recovered by steam distillation (Markham, 1942) after adding 20ml of 7.5 M-NaOH. The methylamine was collected into 20ml of 0.1M-$H_2SO_4$ after distillation of two thirds of the volume. The methylamine hydrogen sulphate was concentrated to 1ml on a rotary evaporator then placed into reaction vessel B (500ml capacity) (Fig. 2.2). Oxidation of methylamine carbon was achieved by wet combustion at 150°C with 40ml of Van Slyke-Folch reagent (Van Slyke and Folch, 1940).

## 2.20 $BaCO_3$ samples from degradations

Discs of Whatman no. 42 filter paper (functional area of 2.204 sq. cm) were used for collecting the $BaCO_3$ precipitates. The discs were washed with 95% ethanol, diethyl ether and weighed before use. $BaCO_3$ precipitates were deposited on the paper discs under suction, washed with $CO_2$ free water, 95% ethanol and diethyl ether. The discs were dried *in vacuo* over $CaCl_2$ then equilibrated at room temperature before weighing on a Mettler H5 micro balance. The dried $BaCO_3$ was then transferred to a scintillation vial and counted.

# CHAPTER 3

## IN VITRO STUDIES OF THE PENTOSE PHOSPHATE PATHWAY

### 3.1 Prefatory comment

If it is assumed that the pentose phosphate pathway is essentially a sequence of reactions whose activity is concerned with biosynthesis, then it follows that hepatic tissues which are actively growing and proliferating should show an increased flux of carbon through the pathway relative to normal liver. One purpose of the experiments described in the following has been to identify those reactions of the pentose pathway at which regulation is exerted and to determine if any alterations in regulation are associated with cell proliferation in the 'growth' tissues. Three general methods have been proposed for the purpose of identifying reactions concerned with regulation (see Rolleston, 1972). The telelogical approach depends on the belief that control should occur early in the pathway and shortly after branch points in order to avoid long stretches of uncontrolled metabolism. Use of this approach simply requires inspection of the pathway and indicates that glucose 6-phosphate dehydrogenase and transketolase are likely candidates for regulation. A second approach is based on the properties of enzymes, on the premise that enzymes exhibiting phenomena such as (1) changed maximum catalytic capacities when dietary or hormonal regimes are manipulated, (2) low activities in comparison with other enzymes of the pathway, or (3) activation or inhibition by other intermediates, represent likely control sites. In

this regard, glucose 6-phosphate dehydrogenase fulfills the criteria as a regulatory site in normal liver (Section 1.2.1). On the basis of this logic, the maximum catalytic capacities of all the enzymes of the pentose phosphate pathway were measured in each of the tissues in an attempt to correlate changes in enzyme activity with potential changes in flux through the pathway. It is of note, however, that there is no obligatory link between these changes in the properties of enzymes observed *in vitro* and the conclusions that they infer and are presumed to apply *in vivo*. This approach only shows that the enzyme has properties consistent with aspects of the theory of metabolic control, not that it actually does control metabolism. In the third method, intact tissue preparations are subjected to various treatments which allow different rates of flow through the metabolic pathway, this is followed by measurement of the steady-state concentration of intermediates and the data used to deduce where sites of metabolic control exist. In the present studies, the "treatments" are partial hepatectomy, neoplastic transformation and foetal growth, all of which result in increased cell proliferation and presumably increased rates of flow through the pentose pathway. Thus the steady-state concentrations of the intermediates of the pentose phosphate pathway were measured and their values analyzed in terms of regulatory site identification in the pathway and their potential influence on changes in pathway flux in each of the tissues under study.

Other studies described in this chapter concern the elucidation of the reaction sequence of the pentose

phosphate pathway in growing hepatic tissues. This work involves the comparative analysis of the carbon balance following ribose 5-phosphate dissimilation by enzyme preparations from the various tissues and measurement of the capacity of these enzymes to form hexose 6-phosphate from the L-type pathway intermediate D-*glycero*-D-*ido*-octulose 1,8 bisphosphate.

## 3.2 Development of transplantable tumours

Age and weight-matched male Buffalo rats (8-10 weeks of age and 150-200g) received implants of either the 7777 or 5123C Morris hepatoma as detailed in Section 2.2. The growth of the tumours then proceeded as follows. After implantation at day 0, a period of time elapsed during which no tumour growth was visible or could be detected by palpatation. When the tumour size exceeded 0.3cm in diameter, however, it was easily palpated. The tumours reached this limit of size as indicated in Table 3.1. After the tumour had grown to this initial palpable size, the growth rate proceeded exponentially (Morris, 1975). The animals had usually developed metastases in the lungs near the end of the transplantation interval, by which time the tumour mass was 35-40g wet weight (see Table 3.1). No gross metastases were visible before this time. The health of the host animals appeared to be normal during the transplantation interval. This was monitored by coat appearance, feeding habits (stomach contents) and response to external stimuli. After this time the coat became dull, the animals lost body weight and gradually became less alert. This condition became chronically worse until death. At this

Table 3.1: Growth rates of transplantable tumours

| (a) Tumour | (b) Palpable | (c) Transplantation Interval | (d) Occurrence of Death of Host |
|---|---|---|---|
| 5123C | 20 days | 2.5 - 3.0 months | 3.0 - 4.0 months |
| 7777 | 10 days | 1.0 - 1.5 months | 1.5 - 2.0 months |

(a) Details of tumours and methods of transplantation are described in Section 2.2.

(b) Tumours became palpable when they had exceeded 0.3cm in diameter.

(c) The tumours were usually transplanted when the tumour had grown to 40 grams wet weight. The transplantation interval was defined as the time required for a tumour to grow to 40 grams wet weight after implantation.

(d) Death of the host usually occurred when the total tumour weight had exceeded 50 grams.

The times indicated refer to the intervals which had elapsed following implantation of the tumours.

time the animals exhibited extreme cachexia often having lost 40-50% of their body weight.

## 3.3 Growth rates of tissues

The four 'growth' tissues included in this study are shown in Table 3.2 in order of their relative rates of growth. The data of Table 3.2 was taken from Knox (1972). It is seen that foetal liver is the most rapidly growing tissue followed by regenerating liver. The growth rates of the two hepatomas are significantly slower by comparison. In view of this data, it was considered that any growth-related alterations in the pentose phosphate pathway might be more easily demonstrated with foetal and regenerating liver than with either of the tumours.

## 3.4 Enzyme activities

The maximum catalytic capacities of all of the enzymes of the F-type and L-type pentose pathways except the phosphotransferase and arabinose 5-phosphate epimerase enzymes were measured using 105,000g supernatant extracts prepared from 17-18 day foetal liver, 24h and 48h post-operative regenerating liver, Morris hepatomas 7777 and 5123C, host livers of the tumour-bearing animals and normal adult rat liver. The activities of these enzymes are given in Table 3.3. The results are also shown as a percentage of normal adult liver activity for comparative purposes and are illustrated in Fig. 3.1. Examination of the literature indicates that there is considerable variability in estimates of the activities of a number of the enzymes listed in Table 3.3, however the values shown here for adult

Table 3.2: Growth rates of foetal and regenerating livers and Morris transplantable hepatomas 7777 and 5123TC

| Tissue | $b_{vol}$ ±SD |
|---|---|
| Foetal liver | 0.218 |
| Regenerating liver | 0.129±0.013 |
| Morris hepatoma 5123TC | 0.013 |
| Morris hepatoma 7777 | 0.030 |
| Adult liver | 0.000 |

The relative growth rates of the above tissues are expressed as $b_{vol}$. The $b_{vol}$ is defined as the $\log_{10}$ of the ratio of the tissue volume (or mass) on day tn+1 to day tn during early linear growth. Rates for foetal liver were calculated from daily weights in 2 series of foetuses from the 14th day of gestation. Rates for regenerating liver were calculated from this tissue's linear exponential growth at 0-2 days post-operatively in 4 published series. all data in this table is taken from Knox (1972).

liver are in good agreement with those reported by Gumaa *et al*. (1969), Baquer and McLean (1972) and Baquer *et al*. (1976). The activities of these enzymes in sham-operated rats were not significantly different from those reported for adult liver (data not shown), these findings are in agreement with the results of Weber *et al*. (1974).

### 3.4.1 Enzymes of the oxidative segment of the pathway

The data of Table 3.3 and Fig. 3.1 show that the maximum catalytic capacity of glucose 6-phosphate dehydrogenase is elevated in all 'growth' tissues examined relative to normal liver. The activity of this enzyme in host livers is not increased and is significantly depressed in the liver of the 7777 tumour-bearing host. Hepatoma 5123C shows the greatest activity followed by hepatoma 7777 > 48h regenerating liver > foetal liver > 24h regenerating liver > normal liver > 5123C host liver > 7777 host liver. Thus it appears that increased activity of glucose 6-phosphate dehydrogenase is associated with cell proliferation, although it does not correlate with growth rate (see Table 3.2). The activity of 6-phosphogluconate dehydrogenase was found to be relatively constant and near normal liver activity in all tissues except foetal liver where it is significantly lower than the value for normal liver. An interesting feature of the alterations in the activities of the two dehydrogenase enzymes is the ratio of 6-phosphogluconate dehydrogenase activity to glucose 6-phosphate dehydrogenase activity. This ratio is 2.63 in adult liver whereas the ratio for foetal liver is 0.28; hepatoma 5123C, 0.36; hepatoma 7777, 0.40; 48h

Table 3.3: The maximum catalytic capacities of enzymes in normal, foetal, regenerating liver and Morris hepatomas 7777 and 5123C and host livers of tumour-bearing rats (nmol/min/mg/30°)

| Enzyme | Tissue | | | |
|---|---|---|---|---|
| | Adult Liver | Foetal Liver | 24h Regenerating Liver | 48h Regenerating Liver |
| Glucose 6-phosphate dehydrogenase | 13.11±1.80(7) | 41.05±1.75 | 28.05±0.62 | 42.84±0.94 |
| 6-phosphogluconate dehydrogenase | 34.55±6.63(7) | 11.60±2.15 | 26.32±2.19 | 35.43±2.84 |
| Rib 5-P isomerase | 37.58±3.21(8) | 43.87±0.50 | 37.96±2.28 | 76.92±1.10 |
| Ru 5-P epimerase | 79.96±7.05(8) | 48.66±3.18 | 82.66±7.52 | 79.76±8.35 |
| Transketolase | 26.51±2.51(8) | 11.51±1.05 | 18.50±0.42 | 14.44±1.40 |
| Transaldolase | 13.56±1.17(5) | 8.48±0.53 | 9.41±0.73 | 14.81±0.95 |
| Aldolase | 41.86±2.00(5) | 35.61±3.57 | 42.06±3.57 | 63.42±3.51 |
| Soluble protein mg/g | 94.2±8.5 | 58.1±0.6 | 65.7±0.6 | --- |

The maximum catalytic capacities of the enzymes above were measured using the 105,000g supernatant extracts prepared as described in Section 2.3. The assay procedures used are referred to in Section 2.6. Protein was determined by the method of Lowry *et al.* (1951). The results are the mean±SEM for the number of determinations shown in parentheses for normal liver and for 5 determinations for all other tissues.

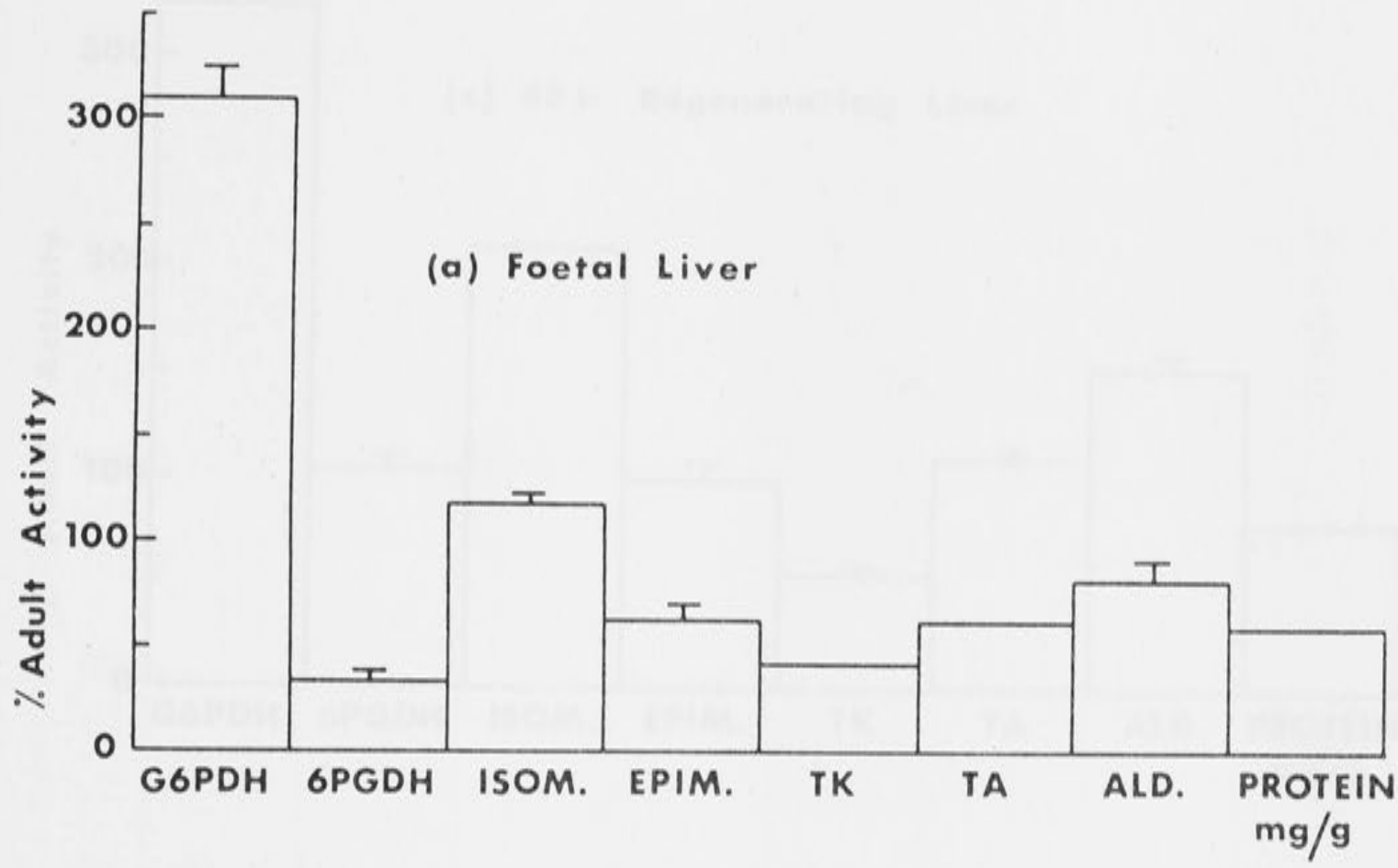
Table 3.3 (continued)

| Enzyme | Hepatoma 7777 | Host Liver 7777 | Hepatoma 5123C | Host Liver 5123C |
|---|---|---|---|---|
| Glucose 6-phosphate dehydrogenase | 98.33±5.38 | 6.82±0.52 | 114.35±17.02 | 14.09±2.13 |
| 6-phosphogluconate dehydrogenase | 39.39±2.42 | 33.51±3.46 | 40.64±5.15 | 20.17±2.19 |
| Rib 5-P isomerase | 8.27±0.75 | 29.31±0.75 | 75.49±6.21 | 71.18±9.98 |
| Ru 5-P epimerase | 155.92±10.39 | 122.33±4.80 | 78.05±8.82 | 78.96±9.41 |
| Transketolase | 33.67±3.45 | 27.57±4.24 | 10.72±1.05 | 17.29±2.36 |
| Transaldolase | 24.99±4.47 | 11.80±0.41 | 8.09±1.62 | 4.75±0.99 |
| Aldolase | 35.16±1.67 | 38.93±2.93 | 34.26±3.09 | 50.74±6.83 |
| Protein mg/g | 66.3±0.7 | 78.2±0.6 | 62.1±0.8 | 80.5±0.9 |

Fig. 3.1: The maximum catalytic capacities of enzymes of the pentose phosphate pathway in 'growth' tissues relative to normal, adult liver

The data of Table 3.3 was used to construct histograms showing the activities of the enzymes in foetal (a), regenerating (b,c) and host (f,g) livers and hepatomas 5123C (e) and 7777 (d) expressed as a percentage of the values measured in adult liver. For details of the assay procedures used see the legend to Table 3.3.

Fig. 3.1


300
200
100
0
% Adult Activity
(a) Foetal Liver
G6PDH
6PGDH
ISOM.
EPIM.
TK
TA
ALD.
PROTEIN
mg/g

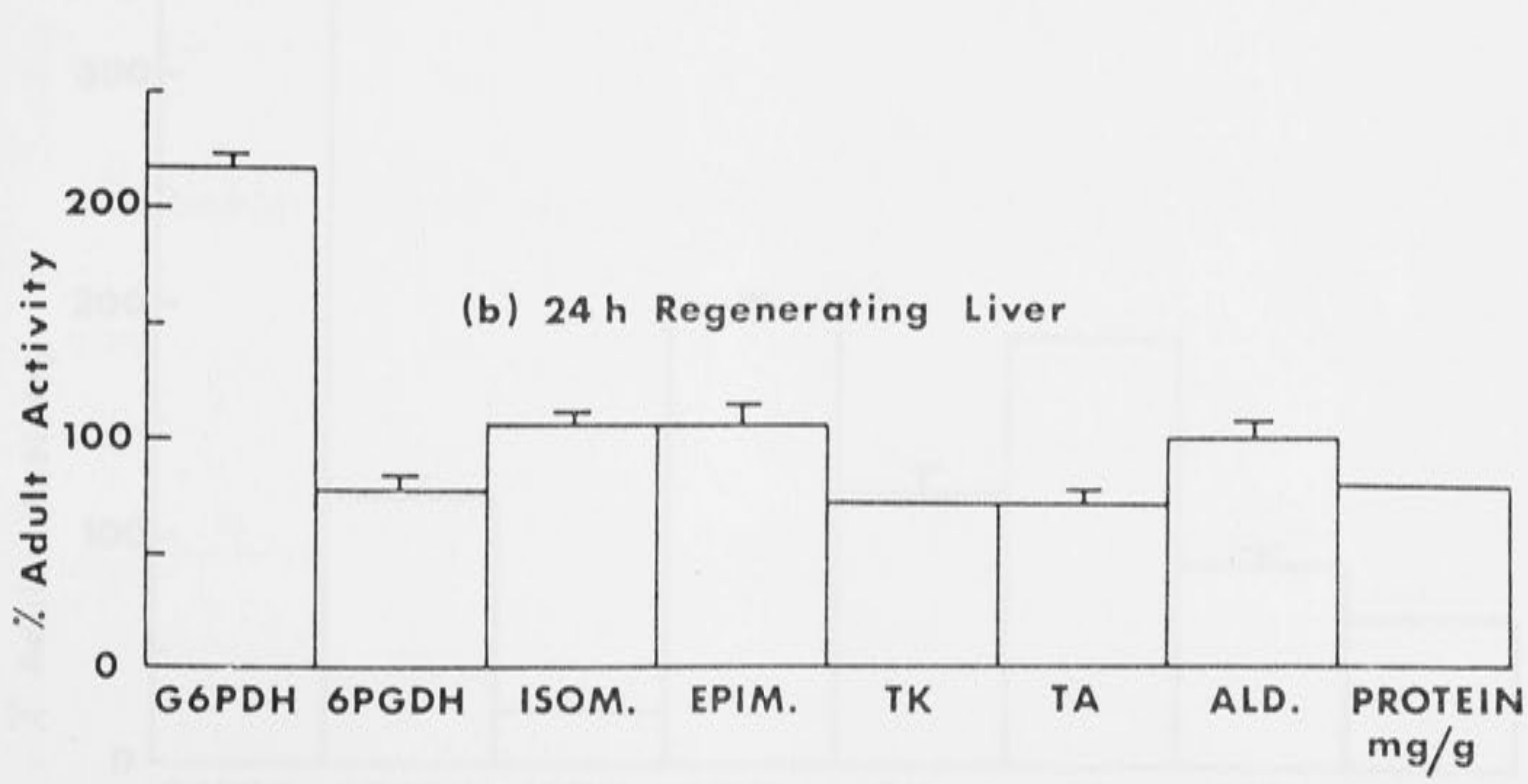

200
100
0
% Adult Activity
(b) 24 h Regenerating Liver
G6PDH
6PGDH
ISOM.
EPIM.
TK
TA
ALD.
PROTEIN
mg/g

Fig. 3.1

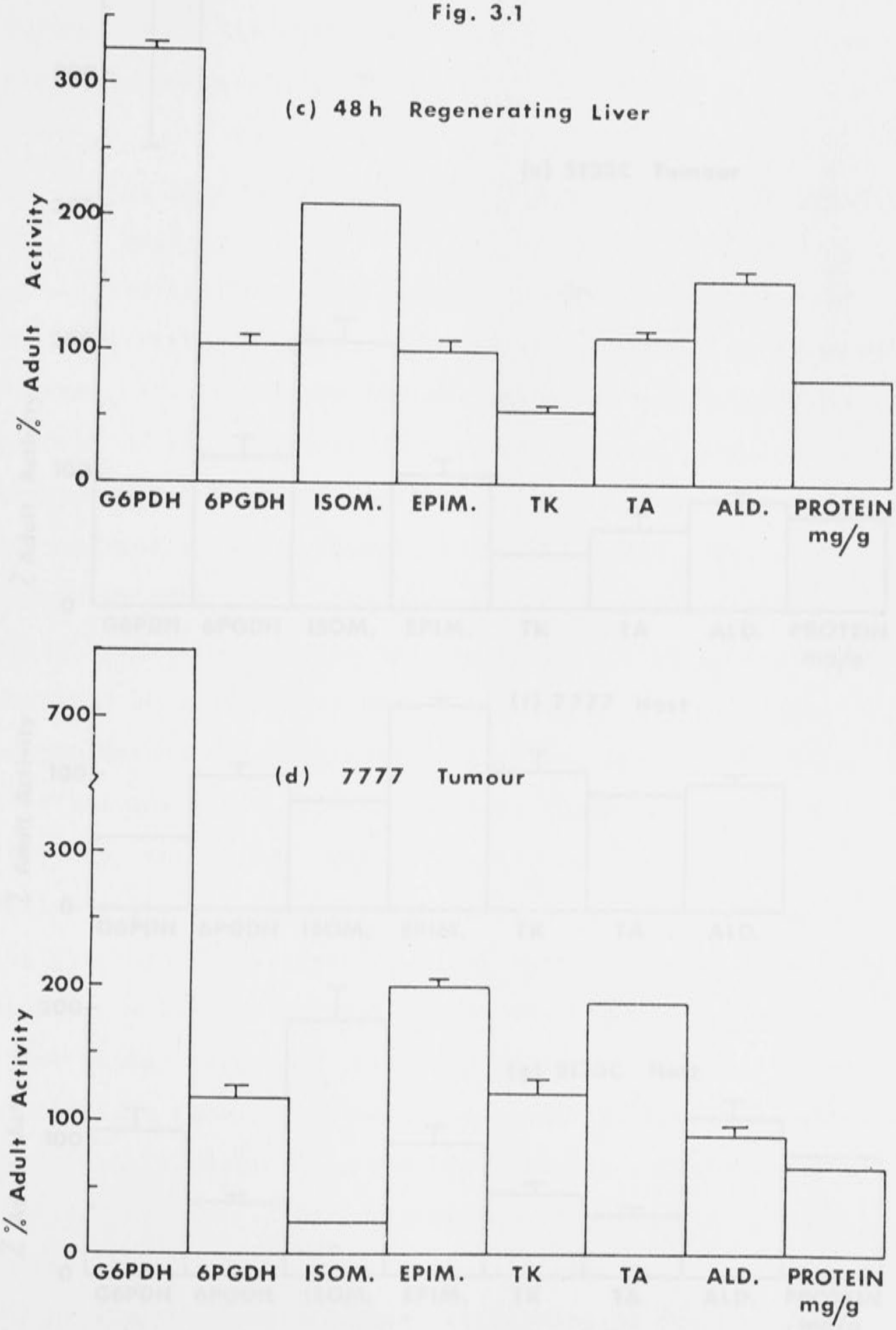
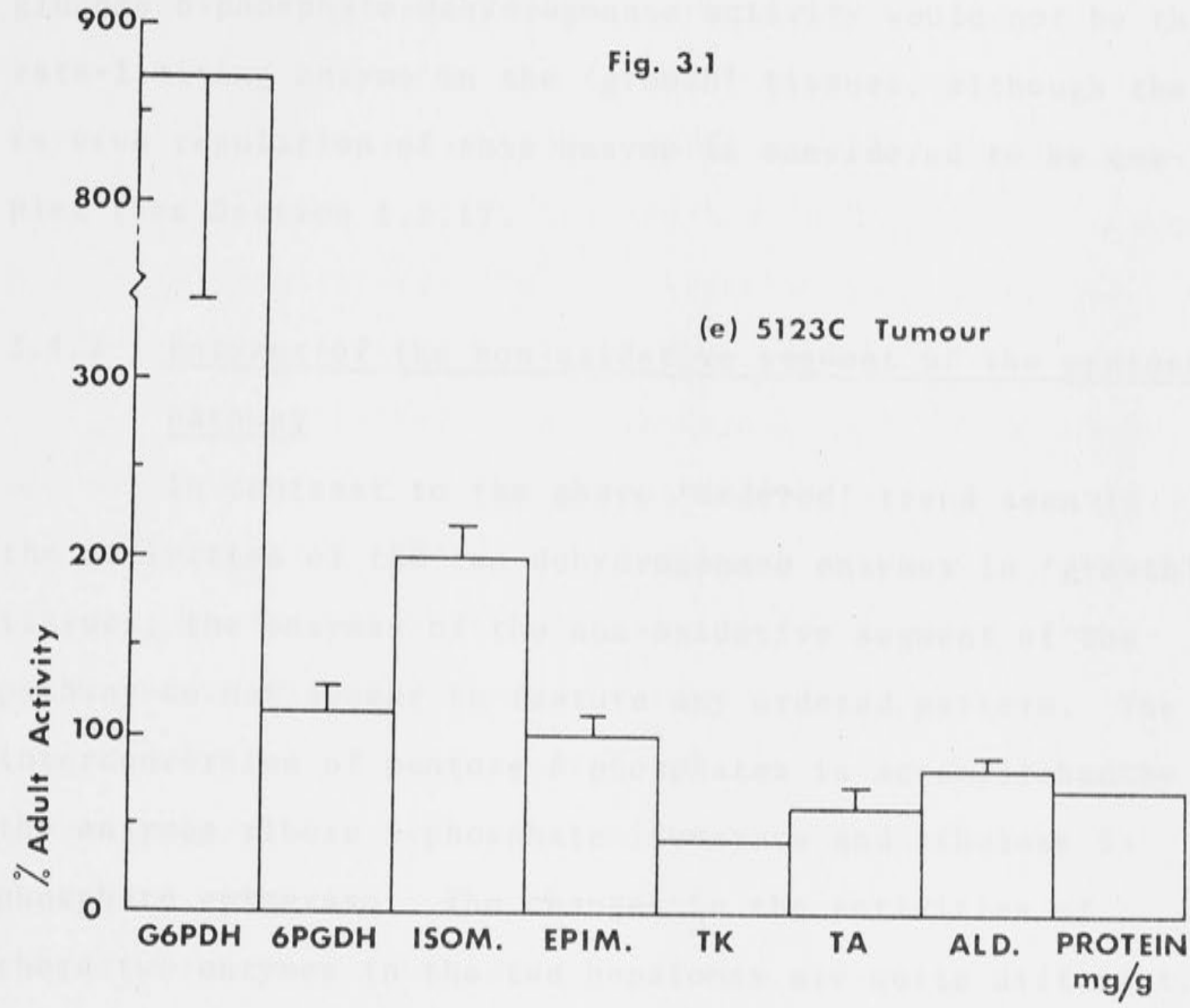
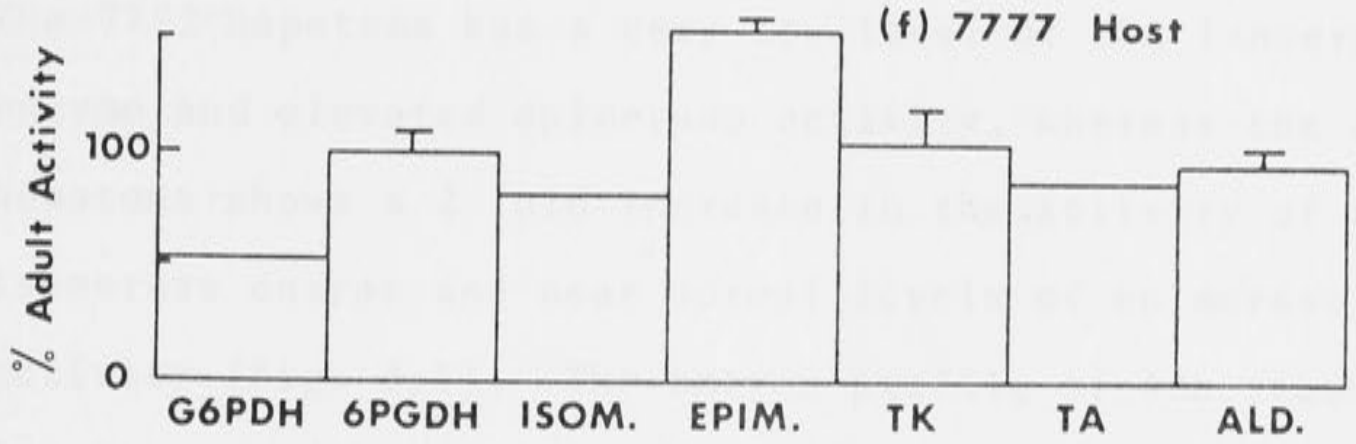

(c) 48 h Regenerating Liver
% Adult Activity
300
200
100
0
G6PDH
6PGDH
ISOM.
EPIM.
TK
TA
ALD.
PROTEIN mg/g
(d) 7777 Tumour
700
300
200
100
0
G6PDH
6PGDH
ISOM.
EPIM.
TK
TA
ALD.
PROTEIN mg/g

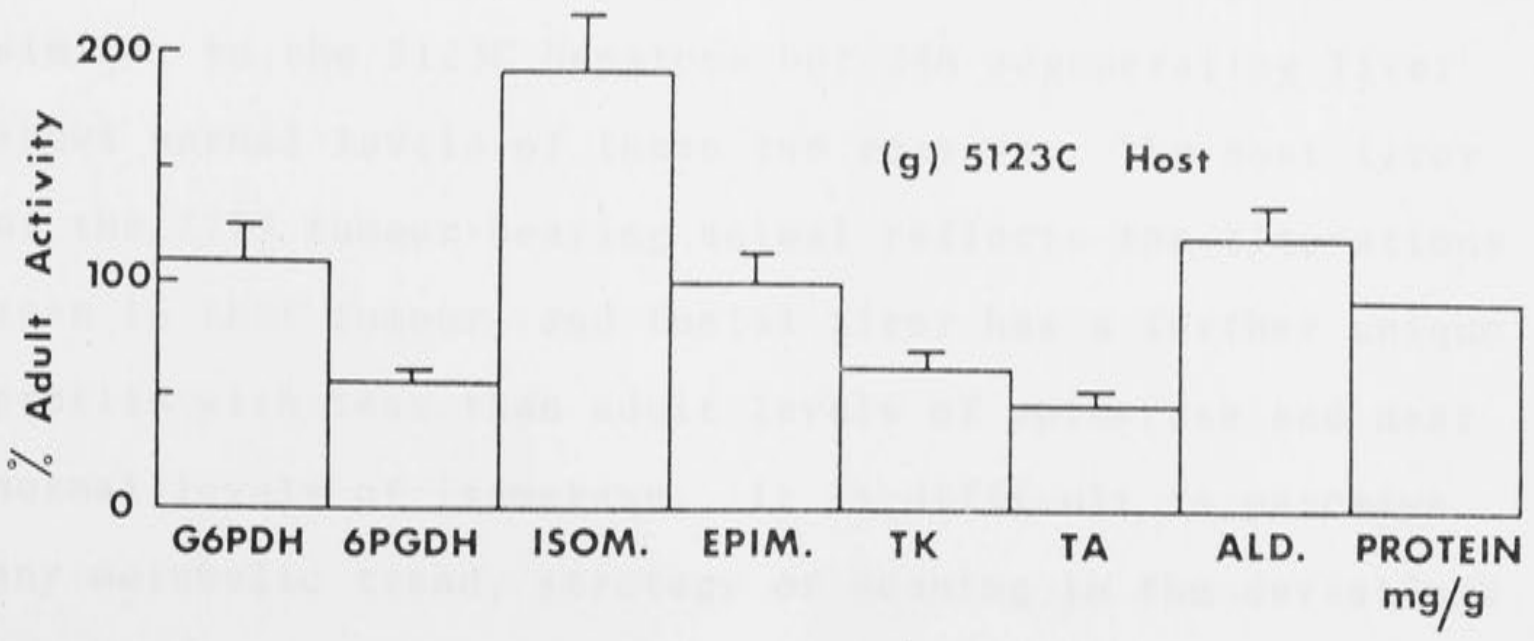
Fig. 3.1




(e) 5123C Tumour




(f) 7777 Host




(g) 5123C Host

regenerating liver, 0.83; and 24h regenerating liver 0.94. Thus on the basis of maximum catalytic capacities alone, glucose 6-phosphate dehydrogenase activity would not be the rate-limiting enzyme in the 'growth' tissues, although the *in vivo* regulation of this enzyme is considered to be complex (see Section 1.2.1).

### 3.4.2 Enzymes of the non-oxidative segment of the pentose pathway

In contrast to the above 'ordered' trend seen in the activities of the two dehydrogenase enzymes in 'growth' tissues, the enzymes of the non-oxidative segment of the pathway do not appear to feature any ordered pattern. The interconversion of pentose 5-phosphates is accomplished by the enzymes ribose 5-phosphate isomerase and ribulose 5-phosphate epimerase. The changes in the activities of these two enzymes in the two hepatomas are quite different. The 7777 hepatoma has a very low level of the isomerase enzyme and elevated epimerase activity, whereas the 5123C hepatoma shows a 2 fold increase in the activity of the isomerase enzyme and near normal levels of epimerase activity (Fig. 3.1). The enzyme profile of 48h regenerating liver and host liver of 5123C tumour-bearing animals is similar to the 5123C hepatoma but 24h regenerating liver shows normal levels of these two enzymes. The host liver of the 7777 tumour-bearing animal reflects the alterations seen in that tumour, and foetal liver has a further unique profile with less than adult levels of epimerase and near normal levels of isomerase. It is difficult to perceive any metabolic trend, strategy or meaning in the deviations

from normal adult levels of these two enzymes in 'growth' tissues. Further, the maximum catalytic capacities of these enzymes are seen to be in excess of the measured activities of the other enzymes of the non-oxidative segment of the pathway (except for 7777 tumour) suggesting that the changes in activity reported here do not influence metabolic flux through the non-oxidative segment of the pathway, i.e. are not rate-limiting.

The activities of transketolase and transaldolase are either decreased or in the normal range in all tissues examined except the 7777 hepatoma which shows a significant increase in transaldolase activity. The increased transaldolase activity in this hepatoma has been previously reported by Weber *et al*. (1974) who also examined a number of other hepatomas (but not the 5123C) and concluded that increased transaldolase activity was characteristic of and specific for hepatic neoplasia. It is of note that both transketolase and transaldolase activities increase in hepatoma 7777 and decrease in hepatoma 5123C, indicating that there is no malignancy-linked trend in the activity of these two enzymes. The activity of aldolase is similar to that seen in normal liver in all of the tissues examined.

### 3.5 Measurement of "*in vivo*" intermediate concentrations

"Freeze clamped" samples of tissues were taken and metabolites were extracted in perchloric acid as described in Section 2.7. Quantitative estimates of the levels of intermediates were made by enzymic analysis (Section 2.8) and are reported in Table 3.4. Plots of the levels of intermediates as a percentage of the values found in normal

adult liver are shown in Fig. 3.2 (a-e). The values in Table 3.4 for normal adult liver are in good agreement with those of Williams *et al*. (1971) using rabbit liver and Baquer *et al*. (1973) and Gumaa *et al*. (1968) using rat liver.

### 3.5.1 Intermediates of the oxidative segment of the pentose pathway

The data of Table 3.4 and Fig. 3.2 show a large variation in the tissue concentrations of glucose 6-phosphate, 6-phosphogluconate and pentose 5-phosphates in the 'growth' tissues relative to normal liver. The only consistent feature observed in all tissues is a depressed (i.e. more reduced) NADP/NADPH ratio (Table 3.5). The concentrations of glucose 6-phosphate in the 'growth' tissues do not follow any pattern. The two most rapidly growing tissues, i.e. foetal liver and 24h regenerating liver, contain approximately 3 times the normal liver concentration of glucose 6-phosphate whereas the hepatoma and host liver values are near normal and the concentration of glucose 6-phosphate in 48h regenerating liver is only 1/2 that of normal liver. The concentration of glucose 6-phosphate in tissues is a function of a number of variables including the rate of glucose phosphorylation, glycogen synthesis and degradation, gluconeogenesis, glycolysis, glucose 6-phosphatase activity, and the activities of the oxidative and non-oxidative segments of the pentose pathway. Thus alterations in the concentration of this intermediate alone in tissues is impossible to interpret in terms of metabolic flux through any of the above pathways.

Table 3.4: Concentrations of metabolites in normal, foetal, regenerating and host liver and hepatoma 5123C (nmole/g fresh weight)

| Metabolite | Normal Liver | Hepatoma 5123C | Host Liver | Foetal Liver | Regenerating Livers | |
|---|---|---|---|---|---|---|
| | | | | | 24H | 48H |
| Glc 6-P | 244±37 | 196±2 | 245±55 | 608±19 | 629±8 | 116±16 |
| 6-PG | 47±9 | 42±7 | 114±13 | --- | 27±3 | 17±1 |
| Ru 5-P | 57±9 | 7±1 | 10±2 | 62±4 | 13±1 | 24±2 |
| Xlu 5-P | 81±15 | 13±2 | 10±1 | 56±7 | 30±3 | 10 |
| Rib 5-P | 381±84 | 23±1 | 83±6 | 394±92 | 185±23 | 120±19 |
| Seh 7-P | 48±10 | 12±1 | 88±8 | 90±7 | 76±2 | 35±6 |
| Tri-P | 75±8 | 43±2 | 46±6 | 83±14 | 38±3 | 29±3 |
| Fru 6-P | 89±11 | 30±2 | 50±13 | 34±3 | 103±14 | 33±3 |
| Fru 1,6-$P_2$ | 54±7 | 49±7 | 19±3 | 45±12 | 21±4 | 12±1 |
| ATP | 1,377±270 | 519±54 | 268±20 | 1,169±160 | 872±30 | 445±40 |
| ADP | 741±140 | 482±15 | 606±37 | 425±36 | 750±60 | 722±80 |
| AMP | 105±30 | 80±26 | 79±8 | 83±4 | 458±41 | 631±28 |
| Pyr | 98±20 | 34±4 | 38±5 | 91±14 | 56±4 | 56±7 |
| Lac | 1,306±161 | 1,493±81 | 322±39 | 3,228±190 | 1,173±59 | 924±34 |
| Mal | 75±8 | 196±81 | 267±83 | 567±15 | 927±46 | 616±70 |

The values shown above represent the concentrations of intermediates which were determined by the enzymic analysis of "freeze clamped" tissues as described in Sections 2.7 and 2.8. Each value is the mean±SEM of at least three determinations.

Fig. 3.2 (a-e): Concentrations of metabolites in foetal, regenerating, and host liver and hepatoma 5123C relative to normal adult liver

The data of Table 3.4 are plotted as a percentage of the normal adult liver concentrations of metabolites for each 'growth' tissue; (a) foetal liver, (b) 24h regenerating liver, (c) 48h regenerating liver, (d) hepatoma 5123C and (e) host liver of hepatoma 5123C tumour-bearing animals. Details of the enzymic analysis are described in Sections 2.7 and 2.8.

Fig. 3.2(a)

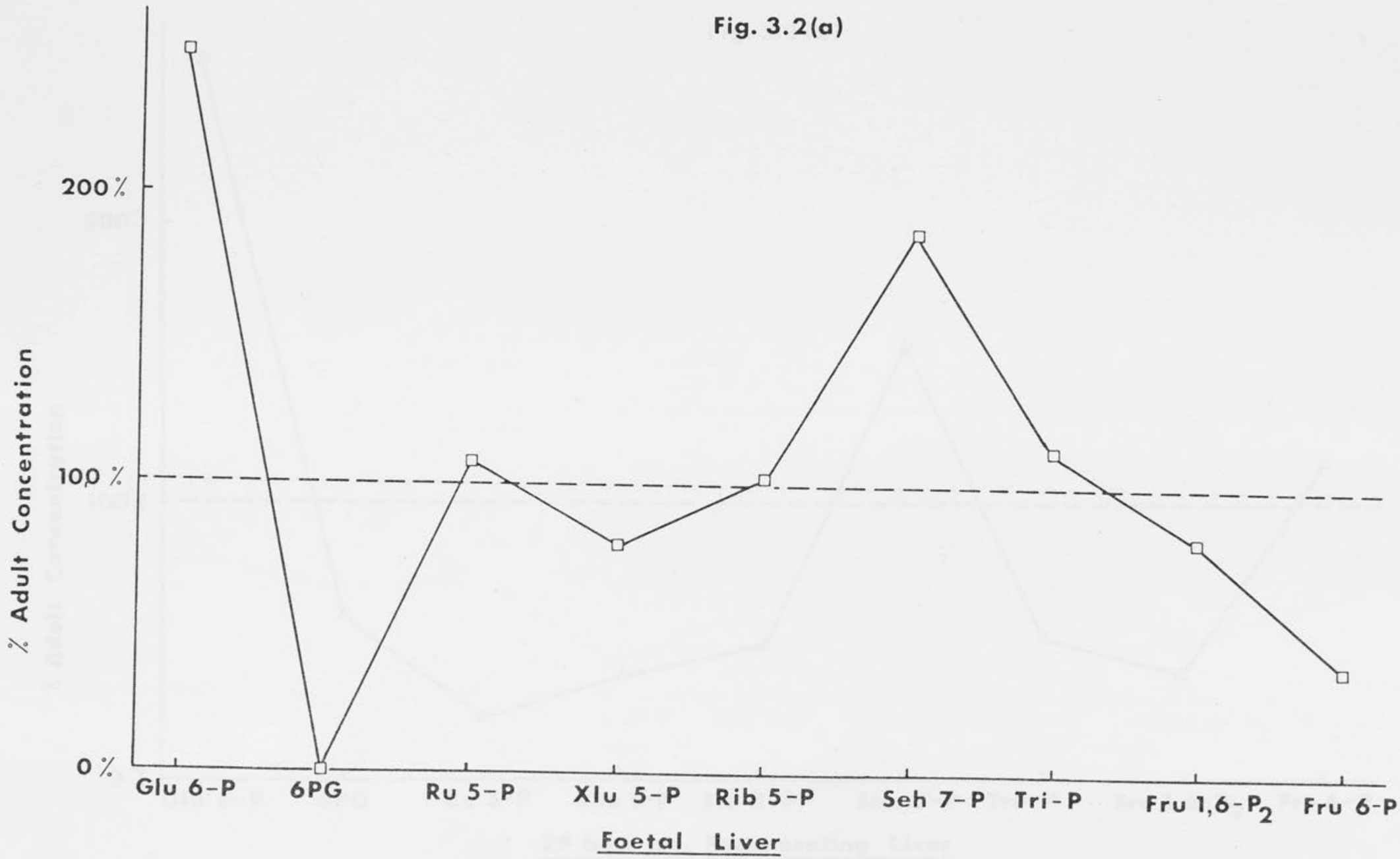

200 %
100 %
0 %
% Adult Concentration
Glu 6-P
6PG
Ru 5-P
Xlu 5-P
Rib 5-P
Seh 7-P
Tri-P
Fru 1,6-P2
Fru 6-P
Foetal Liver

Fig. 3.2 (b)

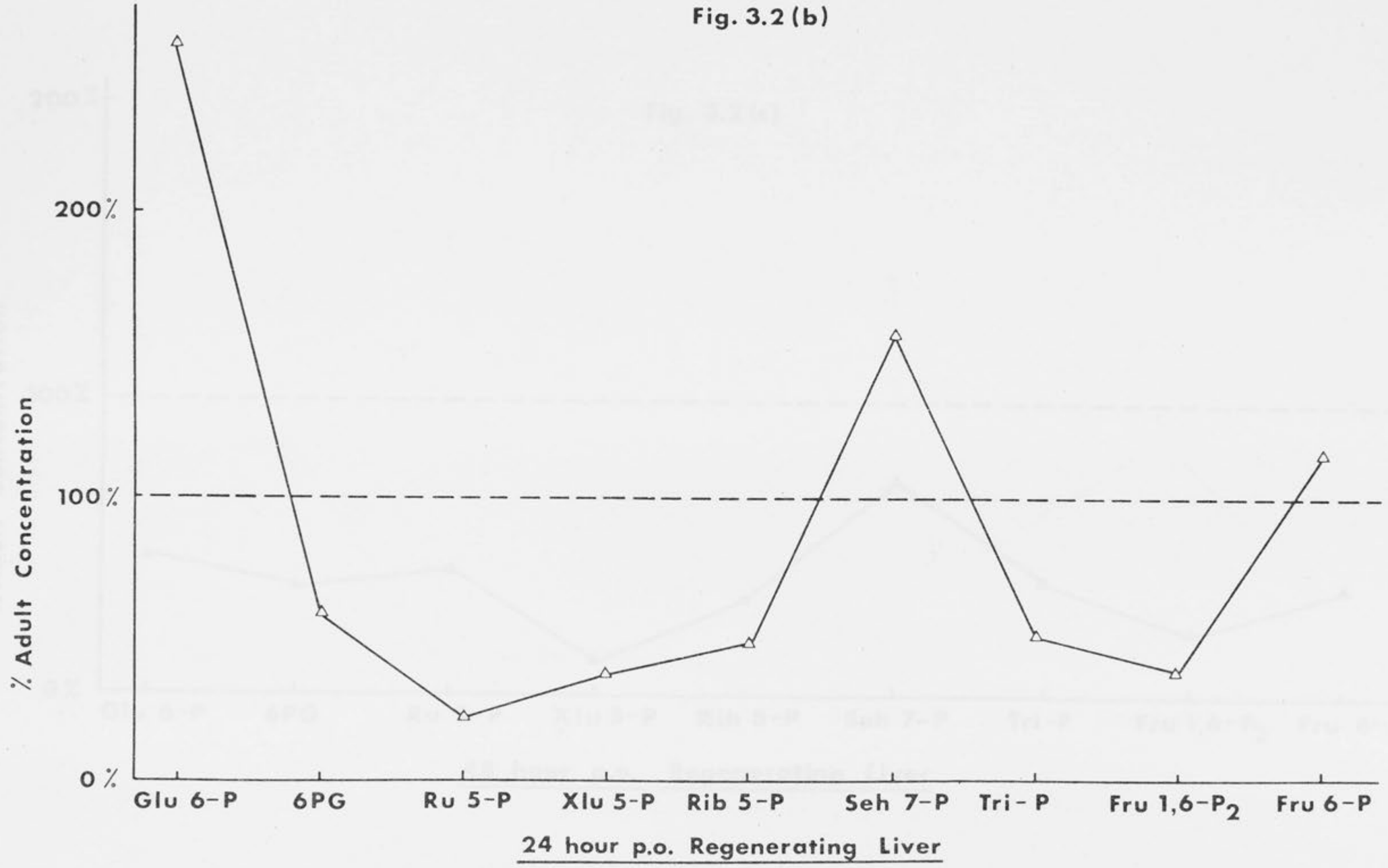
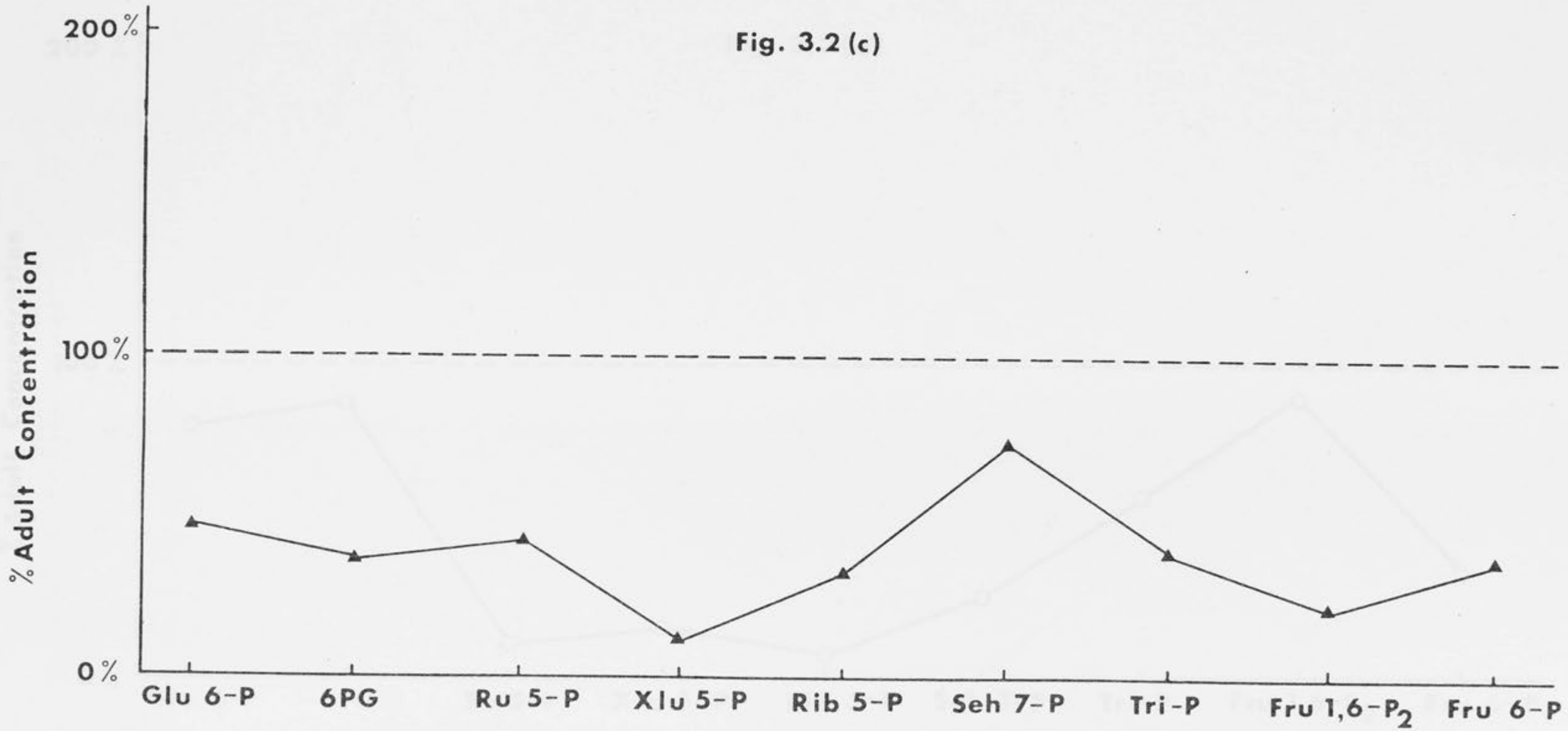
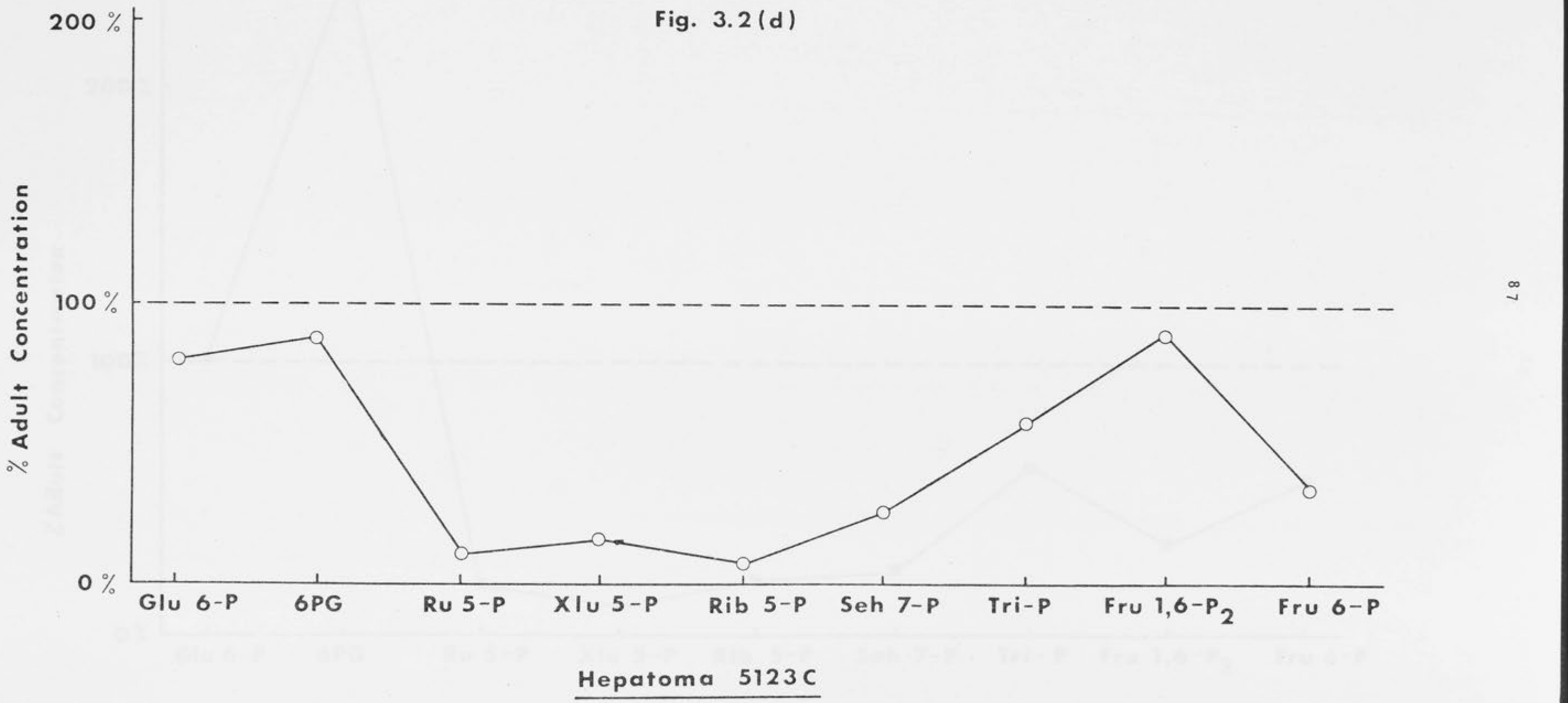

200%
100%
0%
% Adult Concentration
Glu 6-P
6PG
Ru 5-P
Xlu 5-P
Rib 5-P
Seh 7-P
Tri-P
Fru 1,6-P2
Fru 6-P
24 hour p.o. Regenerating Liver

Fig. 3.2 (c)



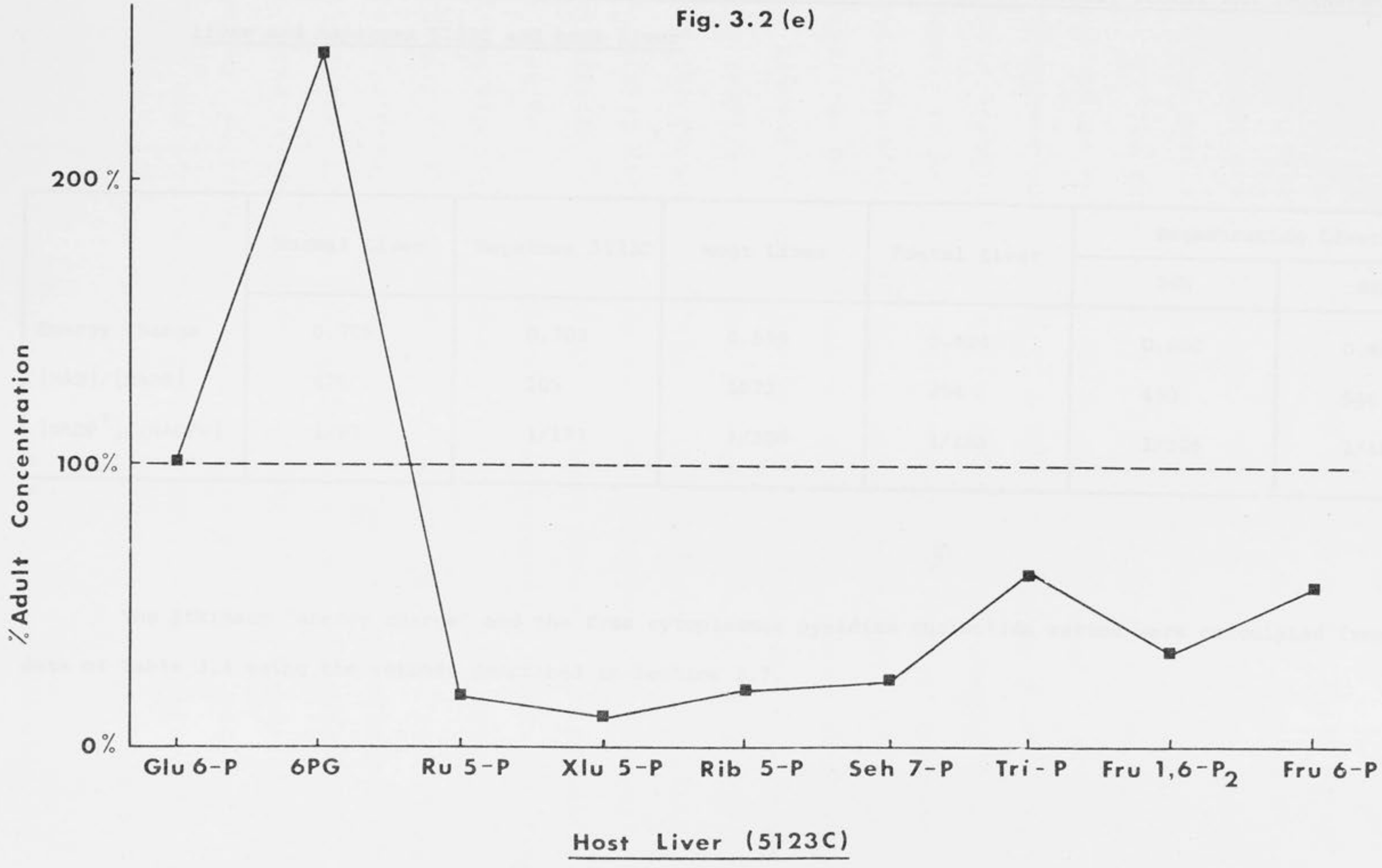

48 hour p.o. Regenerating Liver

Fig. 3.2 (d)




Hepatoma 5123C

Fig. 3.2 (e)




Host Liver (5123C)

Table 3.5: "Energy Charge" and free cytoplasmic pyridine nucleotide ratios of normal, foetal and regenerating liver and hepatoma 5123C and host liver

| | Normal Liver | Hepatoma 5123C | Host Liver | Foetal Liver | Regenerating Livers | |
|---|---|---|---|---|---|---|
| | | | | | 24H | 48H |
| Energy Charge | 0.786 | 0.703 | 0.599 | 0.824 | 0.600 | 0.498 |
| [NAD]/[NADH] | 676 | 205 | 1072 | 254 | 430 | 546 |
| $[NADP^+]/[NADPH]$ | 1/97 | 1/171 | 1/208 | 1/185 | 1/326 | 1/185 |

The Atkinson "energy charge" and the free cytoplasmic pyridine nucleotide ratios were calculated from the data of Table 3.4 using the methods described in Section 2.7.

The steady-state concentration of 6-phosphogluconate is well below normal liver values in all tissues except the hepatoma 5123C which is in the normal range and host liver of the 5123C tumour-bearing animal which shows a marked increase. The concentrations of the pentose 5-phosphates are significantly reduced in all tissues except for the most rapidly growing tissue, foetal liver.

The data of Table 3.4 was used to calculate the mass action ratios for the reactions catalyzed by glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, ribose 5-phosphate isomerase and ribulose 5-phosphate epimerase. These ratios, together with the reported apparent equilibrium constants ($K_{eq}$), are shown in Table 3.6. The comparison of mass action ratios with $K_{eq}$ values shows that the glucose 6-phosphate dehydrogenase catalyzed step is the only reaction far removed from equilibrium and suggests that the regulation of the oxidative segment of the pathway is largely determined by glucose 6-phosphate dehydrogenase activity *in vivo* in all the tissues studied. The regulation of flux by glucose 6-phosphate dehydrogenase occurs in spite of the significant increase in the maximum catalytic capacity of this enzyme in the 'growth' tissues, especially the hepatomas. All other enzymes are near-equilibrium in all tissues and are not considered to be regulatory. Thus in terms of the regulation of the oxidative segment of the pathway, perterbations in metabolism associated with cell growth and proliferation do not alter the major site of regulation of flux from glucose 6-phosphate to pentose 5-phosphate.

It is of interest to compare the intermediate

Table 3.6: Calculated mass action ratios and reported equilibrium constants for some reactions of the pentose phosphate pathway

| Reaction Catalyzed by: | Reported $K_{eq}$ | Normal Liver | Hepatoma 5123C | Host Liver | Foetal Liver | Regenerating Livers | |
|---|---|---|---|---|---|---|---|
| | | | | | | 24H | 48H |
| Glucose 6-phosphate dehydrogenase | $2.7 \times 10^{4}$ (1) | 18.8 | 36.6 | 96.8 | -- | 14.0 | 27.1 |
| 6-phosphogluconate dehydrogenase | 2.3 - 5.0 (1) | 6.7 | 0.2 | 0.2 | -- | 2.0 | 6.3 |
| Rib 5-P isomerase | 3 (2) | 6.7 | 3.3 | 8.3 | 6.4 | 14.2 | 5.0 |
| Ru 5-P epimerase | 3 (2) | 1.4 | 1.9 | 1.0 | 0.9 | 2.3 | 0.4 |
| Transketolase | 1 (1) | 0.1 | 1.7 | 4.9 | 0.3 | 0.5 | 0.8 |

The values for the mass action ratios shown above were calculated from the data of Table 3.4. The apparent equilibrium constants are (1) as quoted by Gumaa and McLean (1969) or (2) from Davies (1961).

concentrations of hepatic 'growth' tissues with those from rat livers following starvation and refeeding a high carbohydrate diet (Gumaa and McLean, 1968). Starvation followed by refeeding a high carbohydrate diet results in a large increase in the maximum catalytic capacity of glucose 6-phosphate dehydrogenase and an increase in hepatic fatty acid synthesis and is therefore similar to the alterations from normal liver seen in the 'growth' tissues. Gumaa and McLean (1968) reported the tissue concentration of glucose 6-phosphate to be approximately 1/2 that of normal liver, the 6-phosphogluconate concentration to be increased by a factor of 1.3, and the levels of pentose 5-phosphates to be near normal values in the livers of these refed rats. These results are in direct contradiction to the intermediate concentrations reported here for 'growth' tissues. There is no correlation between the levels of the pentose pathway intermediates in growing hepatic tissues with those of rats under dietary conditions which could be interpreted to result in increased glucose carbon flux through the pentose pathway. However, since the concentrations of these intermediates are influenced by numerous other factors aside from the pentose pathway, no definite conclusions regarding relative flux through this pathway can be made based on this data alone. The general conclusion to be drawn from this comparison is that stimulation of carbohydrate uptake with an end to fatty acid synthesis has no obvious relationship to metabolic changes observed in the growth-promoted conditions observed here.

### 3.5.2 Intermediates of the non-oxidative segment of the pentose pathway

Measurements of the tissue concentrations of the intermediates of the non-oxidative segment of the pentose pathway are incomplete since erythrose 4-phosphate, the octulose phosphates, heptulose 1,7 bisphosphate and arabinose 5-phosphate cannot be estimated by conventional enzymic analysis. The levels of free monomeric erythrose 4-phosphate are very low in tissues due to the formation of dimers as discussed by Blackmore *et al.* (1976). The tissue concentrations of heptulose 7-phosphate are seen to be well above normal liver values in all tissues except the hepatoma 5123C and 48h regenerating liver both of which are significantly depressed (Table 3.4). The concentration of triose phosphates are well below normal liver values in all tissues except for foetal liver. It is not possible to analyze these alterations in intermediate concentrations in terms of pentose pathway flux. However, it is noteworthy that no growth-related trend is found in the levels of these intermediates indicating that the metabolism of these sugar phosphates is varied and not dominated by demands related to biosynthesis in these 'growth' tissues.

The mass action ratio of the transketolase reaction involving ribose 5-phosphate and xylulose 5-phosphate (Reaction F, Fig. 1.3) is not far removed from equilibrium (Table 3.5) and is not considered to be regulatory. The mass action ratio of the transaldolase reaction of the F-type pathway could not be estimated since the erythrose 4-phosphate concentration cannot be measured in liver (Paoletti *et al.*, 1979b).

### 3.5.3 Adenine nucleotides and pyridine nucleotide ratios

Estimates of the levels of adenine nucleotides were used primarily to determine the success of each individual "freeze clamp" preparation. The averaged results expressed as the "Atkinson energy charge" are shown in Table 3.6. The value for normal liver is in reasonable agreement with those calculated from adenine nucleotide values reported by Bergmeyer (1974). Total concentrations of adenine nucleotides are seen to be significantly lower than normal liver values for the hepatoma, host liver and regenerating liver. The "energy charge" ratios indicate that the freeze clamp procedures reflect to a reasonable degree the *in vivo* concentrations of the intermediates in a reproducible manner.

The free cytoplasmic NAD/NADH ratios are also shown in Table 3.5 for all tissues. These values were measured using the lactate dehydrogenase system as described in Section 2.7. The value for normal liver is in good agreement with that reported by Williamson *et al.* (1967). The values for all tissues indicate a more reduced redox couple except for the 5123C host liver which is significantly more oxidized than normal liver.

## 3.6 Ribose 5-phosphate metabolism

An arbitrary but conventional method of determining the capacity of the enzymes of a tissue to utilize pentose 5-phosphate *via* the non-oxidative segment of the pentose phosphate pathway is the measurement of the rate of hexose 6-phosphate formation from ribose 5-phosphate. The 105,000g dialyzed 'cytosol' supernatant preparation of foetal liver, 24h regenerating and adult liver and of the Morris

hepatomas 7777 and 5123C were incubated with ribose 5-phosphate and enzymic analysis of the reaction products were made at various times as described in Sections 2.8 and 2.9. Table 3.7 (a-e) shows the total amount of carbon in each of the identifiable intermediates at various incubation times. Each value is expressed as μC atom per mg of protein in the incubation. The data are also expressed as plots of the percentage of total carbon in each intermediate over the time-course of the incubation in Figs. 3.3 - 3.5. The rates of ribose 5-phosphate utilization, triose phosphate formation and hexose 6-phosphate formation were calculated from the data of Table 3.7 and are shown in Table 3.8. The amount of triose phosphate shown is the sum of glyceraldehyde 3-phosphate, dihydroxyacetone phosphate and fructose 1,6 bisphosphate. The amount of hexose 6-phosphate is the sum of glucose 6-phosphate and fructose 6-phosphate.

The results of Table 3.8 show the rates of ribose 5-phosphate disappearance to be rapid relative to the rates of triose phosphate and hexose 6-phosphate formation. It is of interest that the extremes in the range of rates of ribose 5-phosphate disappearance occur when enzymes from the two hepatomas are used, with the most rapid rate observed with enzymes from the faster growing 7777 hepatoma. Thus it appears there is no malignancy-linked trend in the capacity of enzymes to catabolize ribose 5-phosphate, at least under these incubation conditions. It should be noted, however, that metabolism of ribose 5-phosphate by phosphoribosylpyrophosphate synthetase (EC 2.7.6.1) (the first enzyme in the utilization of ribose 5-phosphate for

Table 3.7: Carbon-balance studies for the enzyme-catalyzed conversion of ribose 5-phosphate to hexose 6-phosphate

(a) Normal liver enzymes

| Time (min) | 0 | 2 | 6 | 15 | 30 | 60 | 90 | 120 |
|---|---|---|---|---|---|---|---|---|
| Rib 5-P | 9.64±0.85 | 7.21±0.23 | 6.28±0.28 | 4.57±0.28 | 2.62±0.29 | 1.42±0.12 | 1.29±0.12 | 1.44±0.11 |
| Xlu 5-P + Ru 5-P | 0 | 0.74±0.16 | 0.83±0.06 | 0.85±0.08 | 0.63±0.10 | 0.43±0.05 | 0.40±0.02 | 0.37±0.04 |
| Seh 7-P | 0 | 0.30±0.04 | 1.06±0.08 | 2.32±0.19 | 3.78±0.24 | 2.67±0.18 | 2.47±0.41 | 1.67±0.15 |
| Tri-P | 0 | 0.07±0.01 | 0.20±0.03 | 0.51±0.03 | 0.92±0.04 | 0.91±0.06 | 0.80±0.05 | 0.73±0.02 |
| Hexose 6-P | 0 | 0 | 0 | 0.22±0.02 | 0.68±0.08 | 2.91±0.04 | 3.64±0.08 | 4.69±0.28 |
| TOTAL | - | 8.32 | 8.37 | 8.47 | 8.63 | 8.34 | 8.60 | 8.90 |
| % Recovery | 100% | 86% | 87% | 88% | 90% | 87% | 89% | 92% |

The data of Table 3.7 (a-e) show the amount of carbon recovered in each of the intermediates shown above. The results are expressed as µC atoms per mg of protein and represent the means±SEM of 5 determinations for each tissue. The incubation conditions and the methods of analysis of the intermediates are described in Sections 2.9 and 2.7 respectively. The tables (a-e) show the carbon balance for (a) normal liver, (b) foetal liver, (c) 24h regenerating liver, (d) hepatoma 5123C and (e) hepatoma 7777.

Table 3.7 (b): Foetal liver

| Time (min) | 0 | 2 | 6 | 15 | 30 | 60 | 90 | 120 |
|---|---|---|---|---|---|---|---|---|
| Rib 5-P | 9.0±0.3 | 6.90±0.51 | 5.98±0.21 | 4.45±0.19 | 2.46±0.18 | 1.44±0.13 | 1.25±0.10 | 1.24±0.11 |
| Xlu 5-P + Ru 5-P | - | 0.84±0.07 | 1.18±0.20 | 1.05±0.16 | 0.97±0.08 | 0.76±0.13 | 0.51±0.04 | 0.42 |
| Seh 7-P | - | 0.14±0.02 | 0.41±0.03 | 1.06±0.09 | 1.41±0.12 | 1.20±0.09 | 1.05±0.08 | 1.01±0.09 |
| Tri-P | - | 0.05±.01 | 0.14±.01 | 0.38±.04 | 0.51±0.04 | 0.62±0.05 | 0.41±0.03 | 0.26±0.02 |
| Hexose 6-P | - | 0 | 0 | 0.51±0.04 | 1.20±0.14 | 2.45±0.21 | 3.61±0.27 | 4.76±0.39 |
| TOTAL | - | 7.93 | 7.71 | 7.45 | 6.55 | 6.47 | 6.83 | 7.69 |
| % Recovery | | 88% | 86% | 83% | 73% | 72% | 76% | 85% |

Table 3.7 (c): Carbon balance 24h regenerating liver

| Time (min) | 0 | 2 | 6 | 15 | 30 | 60 | 90 | 120 |
|---|---|---|---|---|---|---|---|---|
| Rib 5-P | 8.70±0.04 | 6.49±0.51 | 5.81±0.42 | 4.62±0.31 | 2.75±0.18 | 1.76±0.18 | 1.47±0.18 | 1.39±0.17 |
| Xlu 5-P + Ru 5-P | - | 0.97±0.08 | 1.42±0.12 | 1.04±0.12 | 0.99±0.06 | 0.89±0.07 | 0.72±0.06 | 0.59±0.04 |
| Seh 7-P | - | 0.21±0.01 | 0.67±0.05 | 1.71±0.14 | 1.65±0.14 | 1.21±0.11 | 0.92±0.08 | 0.79±0.07 |
| Tri-P | - | 0.05±0.01 | 0.18±0.02 | 0.50±0.04 | 0.53±0.04 | 0.46±0.03 | 0.34±0.03 | 0.27±0.02 |
| Hexose 6-P | - | 0 | 0 | 0.51±0.04 | 1.08±0.10 | 2.81±0.30 | 3.97±0.28 | 4.85±0.41 |
| TOTAL | 8.70 | 7.72 | 8.08 | 8.38 | 7.00 | 7.13 | 7.42 | 7.89 |
| % Recovery | 100% | 89% | 93% | 96% | 80% | 82% | 85% | 91% |

Table 3.7 (d): Hepatoma 5123C

| Time (min) | 0 | 2 | 6 | 15 | 30 | 60 | 90 | 120 |
|---|---|---|---|---|---|---|---|---|
| Rib 5-P | 9.21±0.05 | 7.19±0.31 | 6.11±0.51 | 4.52±0.46 | 2.50±0.21 | 1.92±0.18 | 1.51±0.10 | 1.46±0.13 |
| Xlu 5-P + Ru 5-P | - | 0.82±.07 | 1.35±0.11 | 1.42±0.18 | 1.12±0.09 | 0.85±0.07 | 0.75±0.07 | 0.68±0.07 |
| Seh 7-P | - | 0.13±0.01 | 0.38±0.04 | 0.99±0.07 | 1.58±0.70 | 1.72±0.15 | 1.51±0.20 | 1.01±0.09 |
| Tri-P | - | 0.04±0.01 | 0.13±0.01 | 0.34±.03 | 0.46±0.03 | 0.54±0.04 | 0.42±0.03 | 0.31±0.03 |
| Hexose 6-P | - | 0 | 0 | 0.32±0.04 | 1.05±0.08 | 2.23±0.21 | 3.81±0.41 | 4.65±0.50 |
| TOTAL | 9.21 | 8.18 | 7.97 | 7.59 | 6.71 | 7.26 | 8.00 | 8.11 |
| % Recovery | 100% | 89% | 87% | 82% | 73% | 79% | 87% | 88% |

Table 3.7 (e): Hepatoma 7777

| Time (min) | 0 | 2 | 6 | 15 | 30 | 60 | 90 | 120 |
|---|---|---|---|---|---|---|---|---|
| Rib 5-P | 8.8±0 | 5.67±.15 | 4.70±.22 | 2.67±.28 | 1.88±.21 | 1.06±.03 | 1.13±.14 | 0.87±.09 |
| Xlu 5-P + Ru 5-P | - | 1.11±.09 | 1.95±.13 | 1.47±.24 | 0.83±.18 | 0.47±.07 | 0.29±.08 | 0.18±.03 |
| Seh 7-P | - | 0.20±0.04 | 0.60±0.09 | 1.52±.11 | 2.79±.27 | 1.75±.16 | 1.75±.16 | 0.79±.10 |
| Tri-P | - | 0.12±0.03 | 0.30±0.04 | 0.75±.01 | 0.83±.08 | 0.95±.02 | 1.10±.11 | 0.83±.03 |
| Hexose 6-P | - | 0 | 0 | 0.4±.07 | 1.68±.22 | 3.08±.38 | 4.20±.26 | 4.38±.49 |
| TOTAL | 8.8 | 7.10 | 7.55 | 6.81 | 8.01 | 7.31 | 8.47 | 7.05 |
| % Recovery | 100% | 81% | 86% | 77% | 91% | 83% | 96% | 80% |

Fig. 3.3: Ribose 5-phosphate utilization by enzymes from normal, foetal, and regenerating liver and hepatomas 5123C and 7777

The data of Table 3.7 (a-e) were used to construct a time-course showing the amount of substrate carbon present as ribose 5-phosphate over the initial 30 min of the incubation period. The amount of carbon as ribose 5-phosphate is expressed as a percentage of the initial substrate carbon. The plots shown are (a) normal liver, ● (b) ○ foetal liver, ▼ 24h regenerating liver, (c) ■ hepatoma 5123C, △ hepatoma 7777.

Fig. 3.3

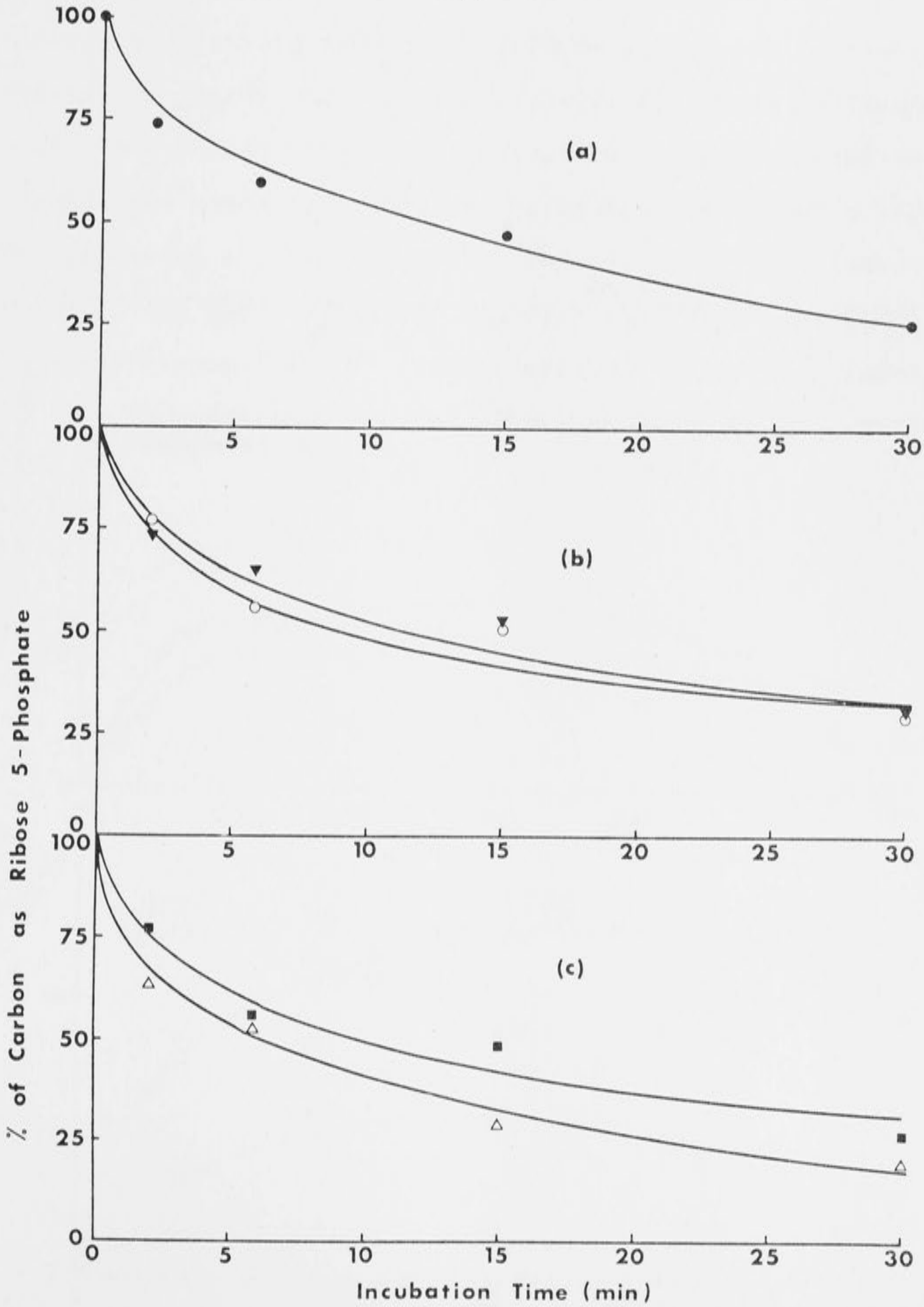

100
75
50
25
0
(a)
100
75
50
25
0
(b)
100
75
50
25
0
(c)
0
5
10
15
20
25
30
% of Carbon as Ribose 5-Phosphate
Incubation Time (min)

Fig. 3.4: The formation of triose-phosphate from ribose 5-phosphate by enzymes of normal, foetal and regenerating liver and hepatomas 5123C and 7777

The data of Table 3.7 (a-e) were used to construct a time-course showing the amount of carbon present as triose phosphate (glyceraldehyde 3-phosphate, dihydroxyacetone phosphate and fructose 1,6 bisphosphate) over the entire 2h of the incubation period. The amount of carbon present as triose phosphate is expressed as a percentage of the initial substrate carbon. The plots shown are (a) ▼ normal liver, (b) ○ foetal liver, ■ 24h regenerating liver, (c) ● hepatoma 5123C, △ hepatoma 7777.

Fig. 3.4

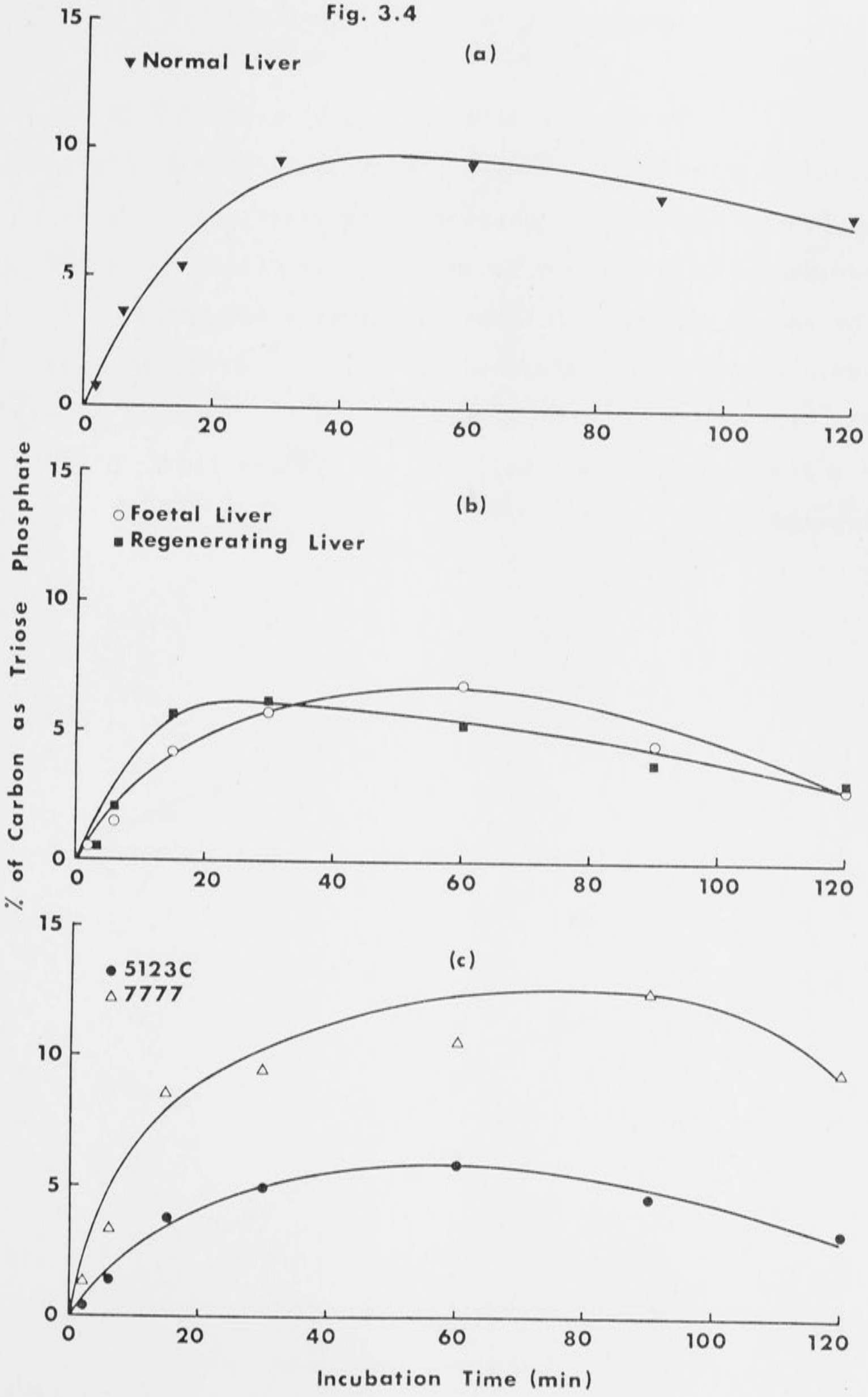

(a)
▼ Normal Liver
15
10
5
0
0
20
40
60
80
100
120
(b)
○ Foetal Liver
■ Regenerating Liver
% of Carbon as Triose Phosphate
(c)
● 5123C
△ 7777
Incubation Time (min)

Fig. 3.5: The formation of hexose 6-phosphate from ribose 5-phosphate by enzymes of normal, foetal and regenerating liver and hepatomas 5123C and 7777

The data of Table 3.7 (a-e) were used to construct a time-course showing the amount of carbon present as hexose 6-phosphate (glucose 6-phosphate and fructose 6-phosphate) over the entire 2h of the incubation period. The amount of carbon present as hexose 6-phosphate is expressed as a percentage of the initial substrate carbon. The plots shown are (a) normal liver, ■ (b) ● foetal liver, □ 24h regenerating liver, (c) ▲ hepatoma 5123C, ○ hepatoma 7777.

Fig. 3.5

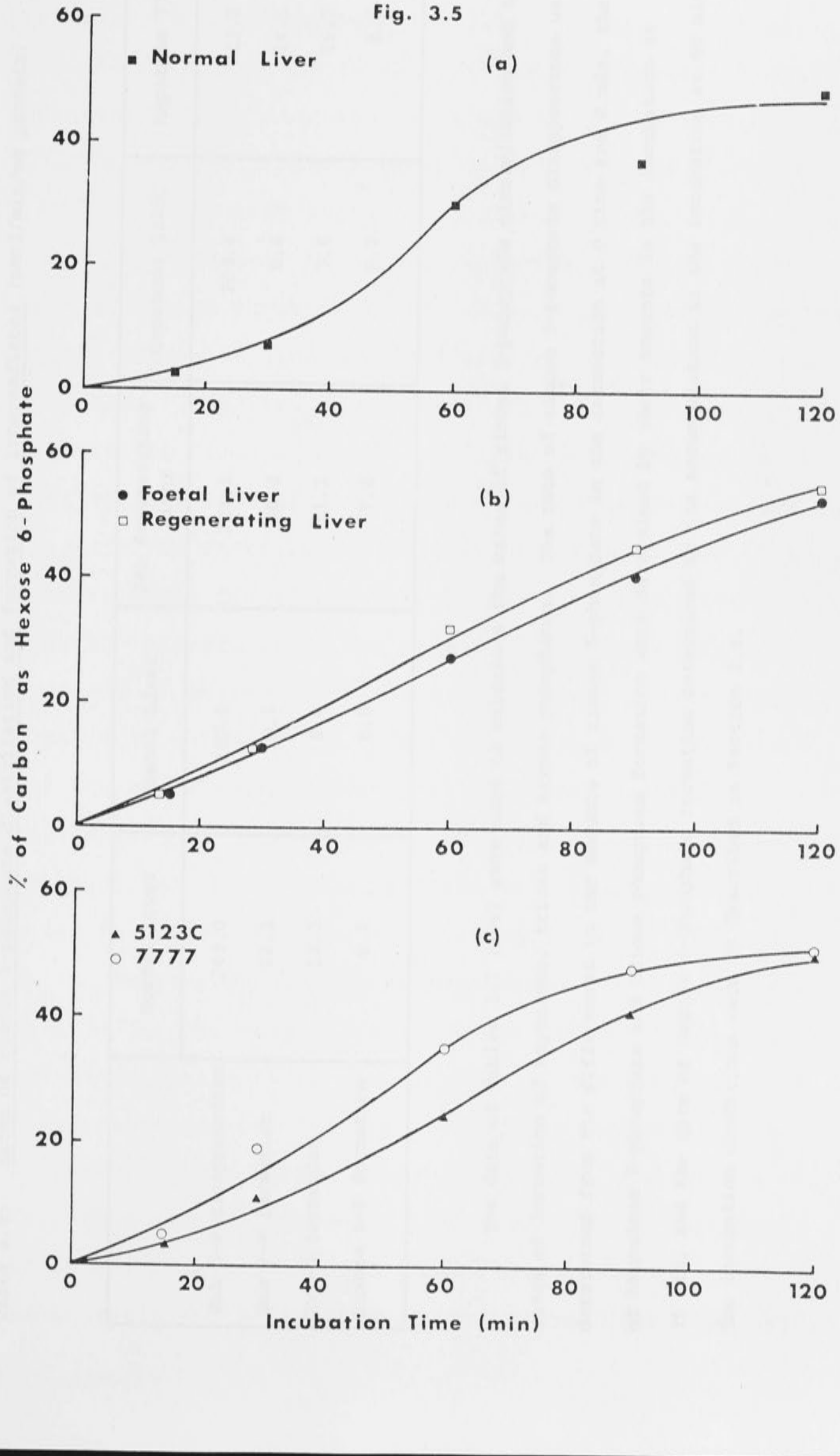

■ Normal Liver
(a)
● Foetal Liver
□ Regenerating Liver
(b)
▲ 5123C
○ 7777
(c)
% of Carbon as Hexose 6-Phosphate
Incubation Time (min)
0
20
40
60
80
100
120

Table 3.8: Rates of ribose 5-phosphate dissimilation and formation of intermediates (nmol/min/mg protein)

| | Normal Liver | Foetal Liver | 24h Regenerating Liver | Hepatoma 5123C | Hepatoma 7777 |
|---|---|---|---|---|---|
| Rib 5-P disappearance | 243.0 | 210.0 | 221.1 | 203.4 | 313.2 |
| Seh 7-P formation | 22.1 | 10.1 | 16.3 | 9.4 | 14.5 |
| Tri-P formation | 11.3 | 8.4 | 11.1 | 7.6 | 16.7 |
| Hexose 6-P formation | 8.1 | 6.8 | 7.8 | 6.2 | 8.6 |



The data of Tables 3.7 (a-e) were used to calculate the rates of ribose 5-phosphate dissimilation and the rates of formation of heptulose, triose and hexose phosphates. The rate of ribose 5-phosphate disappearance was determined from the differences in the amounts of ribose 5-phosphate in the incubation at 0 time and 2 min, the rate of heptulose 7-phosphate and triose phosphate formation were determined by their amounts in the incubation at 15 min, and the rate of hexose 6-phosphate formation determined by its accummulation in the incubation at 60 min. The incubation conditions were as described in Section 2.9.

nucleotide synthesis) is prevented in these experiments due to the absence of ATP. The initial rate of ribose 5-phosphate utilization shown in Table 3.8 was determined from the difference in the concentration of ribose 5-phosphate at zero time and two min. As may be seen in Fig. 3.3, the rate of ribose 5-phosphate disappearance is not linear over the time-course of the incubation.

The rates of heptulose 7-phosphate and triose phosphate formation shown in Table 3.8 were calculated from the concentration of these intermediates at the end of 15 min incubation. The results show that foetal liver, 24h regenerating liver and hepatoma 5123C each have reduced rates of formation of both these sugar phosphates consistent with the decreased activities of transketolase in these tissues. This trend, however, is not characteristic of 'growth' tissues, since the rate of formation of triose-phosphate by enzymes from the 7777 hepatoma are increased relative to normal liver. This observation is consistent with the observed relative rates of ribose 5-phosphate utilization discussed above. The rates of hexose 6-phosphate formation follow the same patterns in tissues as those discussed for triose phosphate formation.

Perhaps the most significant feature of the data of Table 3.7 is the inability to account for a significant percentage of the total carbon added to the incubation in the intermediates of the pentose pathway measured. The deficit of carbon varied somewhat over the time-course of the incubation and between tissues, however at all times and in all tissues, some 10-20% of the initial substrate carbon could not be located in the intermediates measured,

i.e. the intermediates of the F-type pentose pathway. The similarity in the carbon deficit in all tissues with that of normal liver suggests the operation of the L-type pentose pathway in these tissues since this carbon deficit led to the discovery of the new L-type pathway intermediates (Williams *et al.*, 1978b; see Section 1.2.2).

## 3.7 Utilization of D-*glycero*-D-*ido* octulose 1,8 bisphosphate

The capacity of the enzyme preparations from each of the tissues to utilize D-*glycero*-D-*ido* octulose 1,8 bisphosphate for the formation of hexose 6-phosphate was examined primarily as a test for the potential activity of the L-type pentose pathway in these tissues. D-*glycero*-D-*ido* octulose 1,8 bisphosphate and *altro*-heptulose 7-phosphate are substrates for the phosphotransferase reaction (Reaction I, Fig. 1.3). The rate of hexose 6-phosphate formation from these substrates may be seen to represent the activity of this enzyme if it is assumed that the aldolase cleavage of *altro*-heptulose 1,7 bisphosphate (Reaction H, Fig. 1.3) and the transketolase reaction between erythrose 4-phosphate and D-*glycero*-D-*ido* octulose 8-phosphate (Reaction F, Fig. 1.3) are not rate-limiting. The rates of hexose 6-phosphate formation are shown in Table 3.9 for all tissues. The high rate of hexose 6-phosphate formation from *altro*-heptulose 7-phosphate alone was unexpected and is discussed in Chapter 8. Table 3.9 shows that all tissues examined utilize D-*glycero*-D-*ido* octulose 1,8 bisphosphate and *altro*-heptulose 7-phosphate as substrates for hexose 6-phosphate formation. These results are taken to indicate

Table 3.9: Hexose 6-phosphate formation from D-*glycero*-D-*ido*-octulose 1,8 bisphosphate and *altro*-heptulose 7-phosphate by enzymes from normal, 24h regenerating and foetal liver and hepatomas 5123C and 7777

| Substrates | Normal Liver | Foetal Liver | Regen.24h Liver | Hepatoma 7777 | Hepatoma 5123C |
|---|---|---|---|---|---|
| Octulose 1,8-$P_2$ + heptulose 7-P | 3.97 | 4.13 | 5.43 | 6.31 | 4.67 |
| Octulose 1,8-$P_2$ | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Heptulose 7-P | 2.14 | 2.10 | 3.43 | 4.16 | 2.31 |
| Net rate of the phosphotransferase coupled reaction | 1.83 | 2.03 | 2.00 | 2.15 | 2.36 |

Dialyzed 105,000g supernatant extracts prepared as described in Section 2.3 were incubated with D-*glycero*-D-*ido*-octulose 1,8 bisphosphate and/or heptulose 7-phosphate at a concentration of 5 mM and the rate of hexose 6-phosphate determined as described in Section 2.11. The rates are expressed as nmol/min/mg of protein and represent the mean of three determinations for each tissue.

that the phosphotransferase enzyme is active in each of the tissues examined. The activity of the phosphotransferase enzyme has been demonstrated in both directions using buffered acetone dried powders of normal, foetal and regenerating liver (Williams and Cortis, unpublished results).

## 3.8 Discussion

The only consistent adaptive change in the enzyme activity profile observed in 'growth' tissues is the increased maximum catalytic capacity of glucose 6-phosphate dehydrogenase. It is significant that in this regard this enzyme is the only one examined which is far-removed from equilibrium based on the calculated mass action ratios (Section 3.5.1). Thus the only known potential regulatory enzyme in the pathway is increased in activity and indicates an increased capacity for pentose pathway flux in the 'growth' tissues, especially where neoplasia is the growth condition. It is of interest that the only non-normal, non-growth tissue examined, i.e. host liver, does not show an increased activity of glucose 6-phosphate dehydrogenase. The increased maximum catalytic capacity of this enzyme in hepatomas has been reported to be a general charactertistic of hepatic neoplasia (Weber *et al.*, 1974), and has been interpreted in terms of the molecular correlation concept. Briefly, the objective of the molecular correlation concept is the elucidation of the pattern of reprogramming of gene expression in neoplasia as it is manifested in the information content revealed in the activity and behaviour of key enzymes, isoenzymes and metabolic pathways. These key enzymes are categorized as being (i) progression-linked,

i.e. activities correlate with the degree of tumour malignancy; (ii) transformation-linked, i.e. activities increase or decrease in all hepatomas or (iii) coincidental alterations, activities do not relate to malignancy (for a review of the current status of the molecular correlation concept see Weber *et al.*, 1977). With respect to the pentose phosphate pathway, Weber and co-workers argue that reprogramming of gene expression in hepatic neoplasia results in increased glucose carbon flux into pentose 5-phosphate. This proposal is based largely on the increased activities of glucose 6-phosphate dehydrogenase and transaldolase in the spectrum of hepatomas examined by these workers. Both glucose 6-phosphate dehydrogenase and transaldolase are considered to be transformation-linked enzymic markers of malignancy (Weber *et al.*, 1977). The results reported here to do support this proposal for the following reasons: (i) the activity of glucose 6-phosphate dehydrogenase is increased in all growth tissues examined and not just the neoplastic tissues. These results indicate that the enzyme is growth-linked and not specifically transformation-linked; (ii) the activity of glucose 6-phosphate dehydrogenase *in vivo* is under the control of a complex series of regulatory factors apart from the adaptive changes in maximum catalytic capacity measured here (Section 1.2.1). Thus changes in activity measured *in vitro* do not necessarily indicate increased net flux from glucose 6-phosphate to pentose 5-phosphate *in vivo*; (iii) the activity of transaldolase is decreased, not increased, in hepatoma 5123C indicating this enzyme is not transformation-linked; (iv) the role of transaldolase in

the pentose phosphate pathway in hepatic tissues is questionable since it is not an enzyme of the L-type pentose pathway and is known to be inhibited by arabinose 5-phosphate ($K_i$ = 70μM, Williams *et al.*, 1978b). Thus any alterations in the activity of this enzyme may not be reflected in flux through the pentose pathway if the L-type pathway is operative.

There is no evidence to suggest a regulatory site in the non-oxidative segment of the pentose pathway. On the basis of the results reported here, it does not appear that any of the non-oxidative enzymes examined respond in a characteristic fashion to growth situations. Novello *et al.* (1969) examined the effects of alloxan diabetes and insulin treatment, hypothyroidism, hypophysectomy, starvation and diet on the enzymes of the pentose pathway in liver and concluded: "Thus in different hormonal and dietary conditions the response of the pentose pathway varies, no one pattern prevailing". This observation, although failing to enlighten, is consistent with the results reported here. This failure to define or predict a consistent trend in enzyme activities may be due in part to the operation of the L-type pentose cycle in these tissues and the lack of information regarding the properties of the arabinose 5-phosphate epimerase and the phosphotransferase enzymes. Further almost nothing is known about the effects of the L-type pathway intermediates on the catalytic activities of the other enzymes of the pathway. It is considered that any understanding of the regulation of flux through the non-oxidative segment of the pentose pathway must await clarification of these factors.

Studies of ribose 5-phosphate metabolism revealed differences in the rate of ribose 5-phosphate dissimilation, heptulose 7-phosphate formation and triose phosphate formation in the various tissues (Table 3.8). However, there is no growth-related or malignancy-linked trend in these alterations. It is considered that the rates of hexose 6-phosphate formation from ribose 5-phosphate are not significantly different in the 'growth' tissues and therefore that the capacity of the non-oxidative segment of the pentose phosphate pathway as a whole is not altered in these tissues.

The analysis of ribose 5-phosphate metabolism in terms of the carbon balance (Section 3.6) and the measured capacity of the enzymes of growth tissues to utilize D-*glycero*-D-*ido* octulose 1,8 bisphosphate for the formation of hexose 6-phosphate (Section 3.7) provide circumstantial evidence for the operation of the L-type pentose pathway in all tissues examined. It is of note that a similar carbon balance study with the enzymes of rat adipose tissue did not result in the carbon deficit shown in these studies and as such serves as a negative control (Blackmore, 1975).

# CHAPTER 4

# $^{14}$C-ISOTOPE STUDIES WITH ISOLATED CELLS

## 4.1 Prefatory comment

The results of *in vitro* studies such as those described in the preceding chapter can provide only limited information regarding the metabolism of glucose by tissues in general and the role of the pentose phosphate pathway in growth situations in particular. This is due in part to the incomplete knowledge regarding the regulation of the non-oxidative segment of the pathway, and in part to the complexity of the regulation of flux through the oxidative segment. Thus a large increase in the maximum catalytic capacity of the known regulatory enzyme of the pathway (glucose 6-phosphate dehydrogenase) such as that which was observed with both of the Morris hepatomas may not be accompanied by a corresponding increase in glucose 6-phosphate to pentose 5-phosphate flux *in vivo*. The compilation of data such as that presented in the preceding chapter is useful only in support of results from an alternative experimental approach. An alternative approach to investigating glucose metabolism, especially as it relates to the pentose phosphate pathway, involves the use of $^{14}$C-isotopes. The general purpose of the series of experiments presented in this chapter has been to determine if any alterations in the direction and extent of glucose carbon flow occur in neoplastic and regenerating liver tissue by following the fate of $^{14}$C-isotope from specifically labelled glucose as substrate for isolated cells.

Unfortunately the requirement for intact, metabolically viable and homogeneous isolated cell preparations for these studies has resulted in some limitation of the types of tissues which may be examined. Preliminary experiments showed that it was not practical to prepare isolated cells from the solid transplanted hepatomas. Although it was possible to prepare reasonably homogeneous cell preparations by hind quarter perfusion with collagenase followed by density gradient centrifugation using Ficol, the yield of cells prepared in this way was considered too small to be of use. For this reason the 5123TC hepatoma cells in tissue culture were used for all isotope experiments. The 5123TC hepatoma was originally derived from the 5123C transplantable hepatoma used in the *in vitro* work described in the previous chapter. It has been assumed that the enzymic and intermediate profile of these cells is similar to the solid tumour. Attempts to adapt the 7777 hepatoma to tissue culture in this laboratory were unsuccessful. Preliminary experiments also indicated that preparation of isolated foetal liver cells was impractical. The difficulty with foetal liver cells involved both the cell yield and homogeneity since the foetal liver contained a large number of erythropoietic cells. Adapting foetal liver cells to primary cultures to remove erythropoietic cells was not attempted. The results of the *in vitro* studies reported in the preceding chapter indicated no significant alterations in host liver pentose pathway metabolism and it was not considered relevant to continue studies with this tissue. Thus the experiments reported in this chapter and the remainder of the thesis are concerned only with studies

of the 5123TC hepatoma cells, 24h post-operative regenerating liver and normal adult liver cells. The choice of 24h regenerating liver was made since DNA synthesis is reported to be maximal at this time (Bucher, 1963).

It is emphasized that the $^{14}C$-isotope studies reported here were performed primarily to answer questions regarding the relative contribution of the pentose phosphate pathway or cycle and related metabolic events such as nucleic acid and lipid synthesis. It was not considered pertinent to this investigation to determine rates of glycolysis or glycogenolysis etc., although modifications or additions to the experimental procedures could have shed some light on these additional features of glucose metabolism.

## 4.2 Isolated cell preparations

Isolated hepatocytes were prepared by the perfusion of isolated livers from normal, fed rats and 24h post-operative hepatectomized rats with collagenase as described in Section 2.12. As the preparation of isolated cells from liver was a new technique in this department, it was considered necessary to establish that the cell preparations were metabolically viable. Three criteria were applied for this purpose: (1) trypan blue exclusion, (2) the concentration of adenine nucleotides and hexose 6-phosphates measured per g wet weight of cells and (3) the kinetics of $^{14}CO_2$ release over a two hour incubation of the cell preparations with [1-$^{14}C$] and [6-$^{14}C$] glucose.

Freshly prepared hepatocytes from both normal and 24h regenerating liver were consistently greater than 90

and 85% viable respectively as judged by trypan blue exclusion. The cell viability was monitored over a two hour incubation of the cells at 37° and was found to decrease less than 10% over this time-course.

The concentrations of adenine nucleotides, glucose 6-phosphate and fructose 6-phosphate were determined at the end of a 1h incubation of freshly prepared cells at 37° as described in Section 2.7. The concentrations expressed as nmoles/g wet weight are shown in Table 4.1. The results of Table 4.1 are in reasonable agreement with the concentrations of these intermediates in fresh tissue (Section 3.5, Table 3.4). These results suggest that the isolated cell preparations survive a 1h incubation at 37° reasonably intact. Krebs (1969) has shown that liver slices are particularly susceptable to loss of adenine nucleotides and reported values of 500, 350 and 60 nmoles per g of tissue for ATP, ADP, and AMP respectively. Baquer *et al.* (1973) reported values of 1008, 95 and 157 nmoles per g wet weight of cells for ATP, ADP and AMP respectively for isolated hepatocytes after a 1h incubation at 37° in the presence of 20mM glucose. No explanation for the very low levels of ADP was given. It is of note that the increased levels of hexose 6-phosphates in regenerating liver tissue (Table 3.4) are also seen in the isolated cells after a 1h incubation at 37° in the presence of 5mM glucose.

The production of $^{14}CO_2$ following the metabolism of [1-$^{14}$C] and [6-$^{14}$C] glucose by the isolated cell preparations is shown in Table 4.2 expressed as μcarbon atoms of glucose carbon per g of cells per h and in Fig. 4.1 over the time-course of a 2h incubation at 37°. The results

Table 4.1: Intermediate concentrations of isolated hepatocytes

| Intermediate | Concentration (nmoles/g wet weight) | |
|---|---|---|
| | Normal Liver | 24h Regen. Liver |
| ATP | 929±56 | 725±81 |
| ADP | 496±38 | 605±42 |
| AMP | 239±49 | 312±48 |
| Glc 6-P | 309±31 | 625±82 |
| Fru 6-P | 46±5 | 98±18 |

Isolated hepatocytes were prepared by perfusion of isolated livers from normal and 24h regenerating liver with collagenase as described in Section 2.12 and incubated in plastic vials in an oscillating water bath (180 o.p.m., $37^{o}$) for 1h in Krebs-Ringer bicarbonate buffer, gas phase $O_2$:$CO_2$, 95:5; with glucose 5mM final concentration. The incubation was terminated by centrifuging the cells gently and removing the supernatant fluid followed by the addition of ice cold $HClO_4$ as described in Section 2.7. The acid extract of the cells was neutralized with KOH solution and the concentrations of adenine nucleotides and hexose 6-phosphates were determined by enzymatic analysis (Section 2.8). The results are expressed as nmoles/g wet weight of cells and are the mean±SEM of 4 determinations.

indicate that production of $^{14}CO_2$ from both labelled substrates is nearly linear over the two hour incubation for all three cell preparations. These results show that each of the cell preparations were metabolically viable under these conditions with respect to glucose metabolism.

## 4.3 The incorporation of $^{14}C$ into $CO_2$ from [1-$^{14}C$] and [6-$^{14}C$] glucose

The results of Table 4.2 show the incorporation of $^{14}C$-isotope into $CO_2$ from [1-$^{14}C$] and [6-$^{14}C$] glucose by isolated cells from normal and 24h post-operative regenerating liver and hepatoma 5123TC. The results for normal liver are in reasonable agreement with those of Baquer *et al*. (1973, 1976) using liver slices although these workers used glucose at a concentration of 20mM whereas the experiments reported here were performed with the "physiological" glucose concentration of 5mM. It is of interest to compare the ratio of $^{14}CO_2$ production from [1-$^{14}C$] glucose relative to [6-$^{14}C$] glucose (C-1/C-6 ratio). In hepatocytes from normal liver, the C-1/C-6 ratio is 1.9 (Table 4.2). Baquer *et al*. (1973) reported a value of 1.5 for liver slices but a much higher ratio of 7.1 for isolated hepatocytes following incubation at 37$^{o}$ for 1h with 20mM glucose. The increased ratio in hepatocytes was a result of both an increased production of $^{14}CO_2$ from [1-$^{14}C$] glucose and a decreased production of $^{14}CO_2$ from [6-$^{14}C$] glucose relative to liver slices. A repeat of the experiments of Baquer *et al*. (1973) failed to confirm this high C-1/C-6 ratio in the isolated cell preparations used in this study. The addition of phenazine methosulphate (PMS) relieves the

Table 4.2: The conversion of [1-$^{14}$C] and [6-$^{14}$C] glucose into $^{14}CO_2$ by isolated cells from normal and 24h regenerating liver and hepatoma 5123TC

Isolated hepatocytes from normal and 24h regenerating liver were prepared by the perfusion of isolated livers with collagenase as described in Section 2.12. Cell suspensions of hepatoma 5123TC cells were prepared after harvesting the cells from tissue culture (Section 2.13). The cells were incubated in Krebs-Ringer Bicarbonate buffer, pH 7.4 containing 5mM glucose, 0.2% fatty acid free-BSA and 10.0mM HEPES at a concentration of approx. 100mg wet weight of cells per ml, except the hepatoma cells which were incubated in growth media (Section 2.13). The incubations were in glass scintillation vials with a centre well containing 0.5ml of 2N NaOH. The vials were sealed with rubber stoppers (Suba seals). The volume of cell suspension was 3.0ml to which $^{14}$C-labelled glucose was added (approx. 0.2 µCi, specific radioactivity of [1-$^{14}$C] glucose was 24,450 d.p.m./µmole and of [6-$^{14}$C] glucose 30,395 d.p.m./µmole). The incubations were at 37$^{o}$C in a shaking water bath and were terminated by the addition of 1ml of 2N HCl through the Suba seals. The incubation was then continued for a further hour and a sample of the NaOH solution taken for analysis of $^{14}CO_2$ by liquid scintillation counting. The results are expressed as µgram atom of glucose carbon per h per gram wet weight of cells. The results represent the average ±SEM of 5 determinations for each cell type.

Table 4.2: The conversion of [1-$^{14}$C] and [6-$^{14}$C] glucose into $^{14}CO_2$ by isolated cells from normal and 24h regenerating liver and hepatoma 5123TC

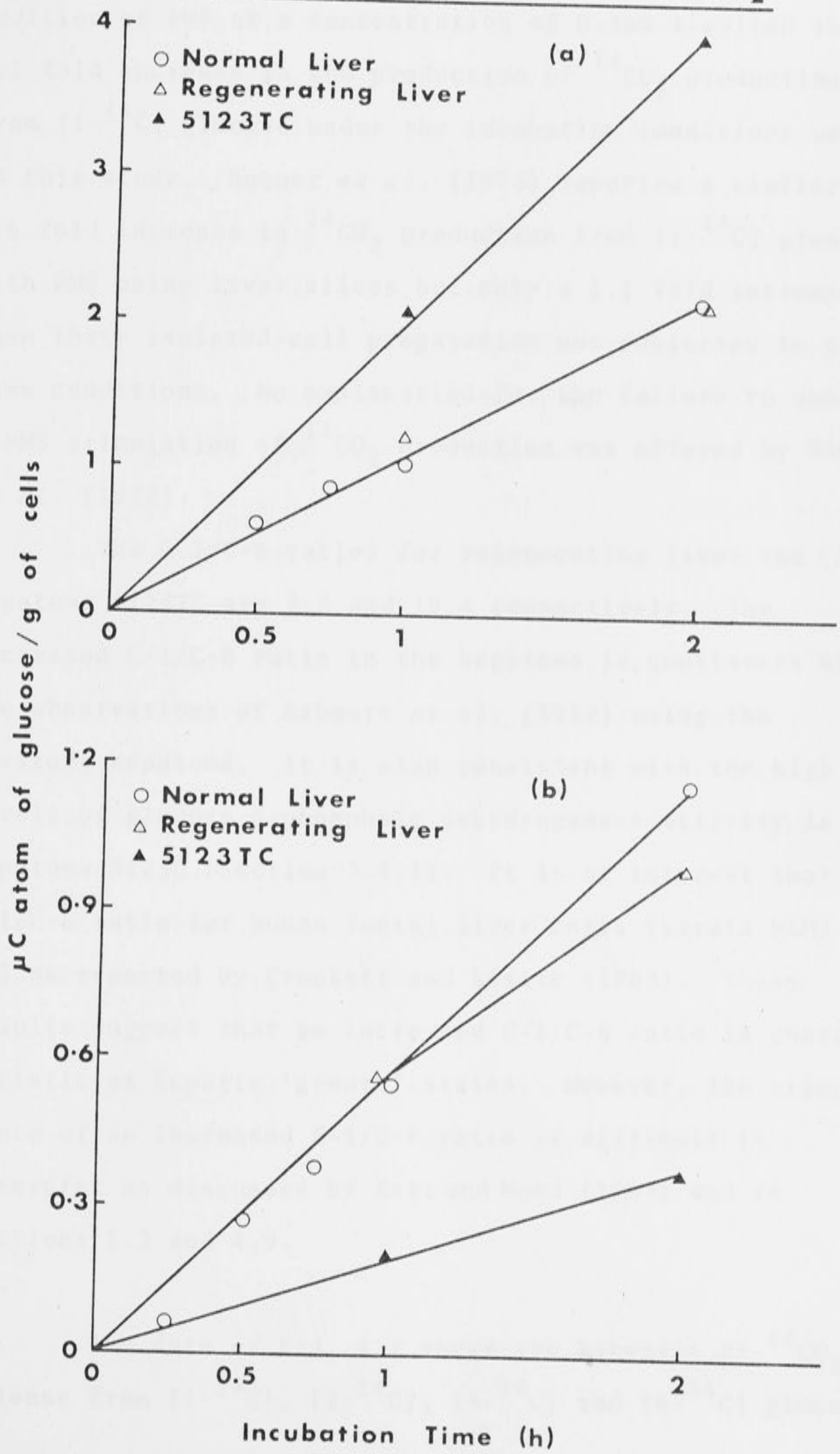
| Cell | [1-$^{14}$C] Glucose | [6-$^{14}$C] Glucose | C-1/C-6 |
|---|---|---|---|
| Normal Liver | 1.21±0.08 | 0.65±0.04 | 1.86 |
| 24h Regenerating Liver | 1.43±0.07 | 0.66±0.05 | 2.17 |
| Hepatoma 5123TC | 2.08±0.12 | 0.20±0.03 | 10.40 |

Fig. 4.1: The incorporation of $^{14}C$ into $CO_2$ following the metabolism of [1-$^{14}C$] and [6-$^{14}C$] glucose by isolated hepatocytes from normal and 24h regenerating liver and hepatoma 5123TC cells

The amount of $^{14}C$-isotope collected as $^{14}CO_2$ is shown. The results are expressed as μC atom of glucose carbon per g wet weight of cells. Details of the incubation conditions and collection of $^{14}CO_2$ are described in the legend to Table 4.2 except that $Ca^{++}$ was omitted from the Krebs-Ringer Bicarbonate buffer in these experiments. The use of $Ca^{++}$- free buffer was employed to allow for a direct comparison of this data with that of Baquer *et al.* (1973). Plot A is the kinetics of $^{14}CO_2$ production from [1-$^{14}C$] glucose and B is [6-$^{14}C$] glucose. The cell preparations used are normal liver cells (○), 24h regenerating liver cells (△), and hepatoma 5123TC cells (▲). The results shown are the average of three determinations in duplicate.

Fig. 4.1

$^{14}C$ isotope incorporation into $CO_2$



NADPH-induced inhibition of glucose 6-phosphate dehydrogenase and therefore its presence is expected to stimulate the production of $^{14}CO_2$ release from [1-$^{14}$C] glucose. The addition of PMS at a concentration of 0.1mM resulted in a 3.2 fold increase in the production of $^{14}CO_2$ production from [1-$^{14}$C] glucose under the incubation conditions used in this study. Baquer *et al*. (1973) reported a similar 3.6 fold increase in $^{14}CO_2$ production from [1-$^{14}$C] glucose with PMS using liver slices but only a 1.1 fold increase when their isolated cell preparation was subjected to the same conditions. No explanation for the failure to observe a PMS stimulation of $^{14}CO_2$ production was offered by Baquer *et al*. (1973).

The C-1/C-6 ratios for regenerating liver and hepatoma 5123TC are 2.2 and 10.4 respectively. The increased C-1/C-6 ratio in the hepatoma is consistent with the observations of Ashmore *et al*. (1958) using the Novikoff hepatoma. It is also consistent with the high levels of glucose 6-phosphate dehydrogenase activity in hepatoma 5123C (Section 3.4.1). It is of interest that the C-1/C-6 ratio for human foetal liver cells (strain HLM) is 3.5 as reported by Crockett and Leslie (1963). These results suggest that an increased C-1/C-6 ratio is characteristic of hepatic 'growth' states. However, the significance of an increased C-1/C-6 ratio is difficult to interpret as discussed by Katz and Wood (1963) and in Sections 1.3 and 4.9.

The data of Fig. 4.2 shows the kinetics of $^{14}CO_2$ release from [1-$^{14}$C], [2-$^{14}$C], [5-$^{14}$C] and [6-$^{14}$C] glucose

by isolated hepatocytes from normal rats. The linearity of $^{14}CO_2$ release is taken to reflect the metabolic viability of these cell preparations. The significant feature of the results shown in Fig. 4.2 is the greater rate of $^{14}CO_2$ production from [2-$^{14}$C] glucose compared with [5-$^{14}$C] glucose. This observation may be interpreted in two ways. If metabolism of glucose 6-phosphate occurs *via* the F-type pentose cycle, $^{14}CO_2$ production from [2-$^{14}$C] glucose would be expected to be greater than that from [5-$^{14}$C] glucose since $^{14}$C-isotope from carbon 2 of glucose is redistributed into carbon 1 of reformed glucose 6-phosphate *via* this reaction sequence (see Fig. 1.2) and will be lost as $^{14}CO_2$ on recycling. Alternatively, if the L-type pentose cycle operates in these cells, $^{14}CO_2$ production would still be predictably greater from [2-$^{14}$C] glucose since this reaction sequence results in $^{14}$C-isotope distribution into C-1 and C-3 of triose phosphate (Fig. 1.3) which would then be released as $^{14}CO_2$ *via* pyruvate decarboxylation and the tricarboxylic acid cycle. Baquer *et al.* (1973) reported the formation of $^{14}CO_2$ from [1-$^{14}$C] pyruvate to be approx. three times that of [2-$^{14}$C] pyruvate by both isolated hepatocytes and liver slices.

## 4.4 Glucose utilization by isolated cell preparations

The concentration of glucose in the incubation

Fig. 4.2

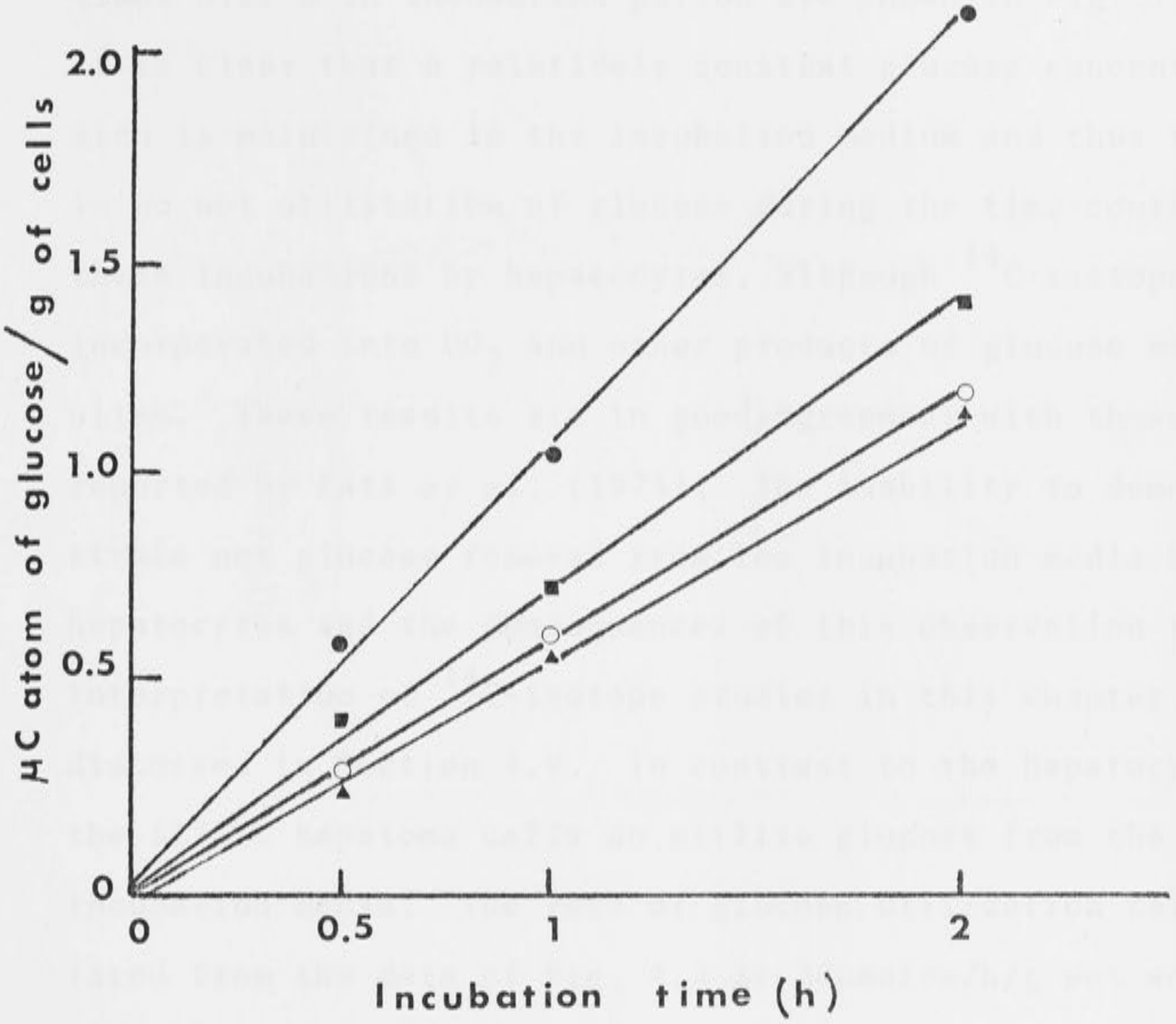



Fig. 4.2: The incorporation of $^{14}C$ into $CO_2$ following the metabolism of [1-$^{14}C$], [2-$^{14}C$], [5-$^{14}C$] and [6-$^{14}C$] glucose by isolated hepatocytes from normal liver

The amount of $^{14}C$-isotope collected as $^{14}CO_2$ following the metabolism of specifically labelled glucose by isolated hepatocytes are shown. The incubation conditions are described in the legend to Fig. 4.1. The $^{14}C$-labelled glucose used are (●), [1-$^{14}C$] glucose; (■), [2-$^{14}C$] glucose; (○), [5-$^{14}C$] glucose; (▲), [6-$^{14}C$] glucose. The results shown are the average for three determinations in duplicate.

media of isolated hepatocytes from normal and 24h post-operative regenerating liver and hepatoma 5123TC at various times over a 2h incubation period are shown in Fig. 4.3. It is clear that a relatively constant glucose concentration is maintained in the incubation medium and thus there is no net utilization of glucose during the time-course of these incubations by hepatocytes, although $^{14}C$-isotope is incorporated into $CO_2$ and other products of glucose metabolism. These results are in good agreement with those reported by Katz *et al.* (1975). The inability to demonstrate net glucose removal from the incubation media by hepatocytes and the consequences of this observation to the interpretation of $^{14}C$-isotope studies in this chapter are discussed in Section 4.9. In contrast to the hepatocytes, the 5123TC hepatoma cells do utilize glucose from the incubation media. The rate of glucose utilization calculated from the data of Fig. 4.3 is 30μmoles/h/g wet weight of cells and is seen to be nearly linear with respect to time over the 2h incubation period.

## 4.5 The incorporation of $^{14}C$ into lipid from [1-$^{14}C$] and [6-$^{14}C$] glucose

The utilization of glucose carbon for the synthesis of lipid by cells from normal and 24h regenerating liver and hepatoma 5123TC was investigated by measuring the rate of incorporation of $^{14}C$-isotope into the lipid fraction of cells following a 2h incubation at 37° with [1-$^{14}C$] and [6-$^{14}C$] glucose. The incubation conditions and isolation of the lipid fraction have been described in Section 2.12. The results are shown in Table 4.3. The rate of

**Fig 4.3**

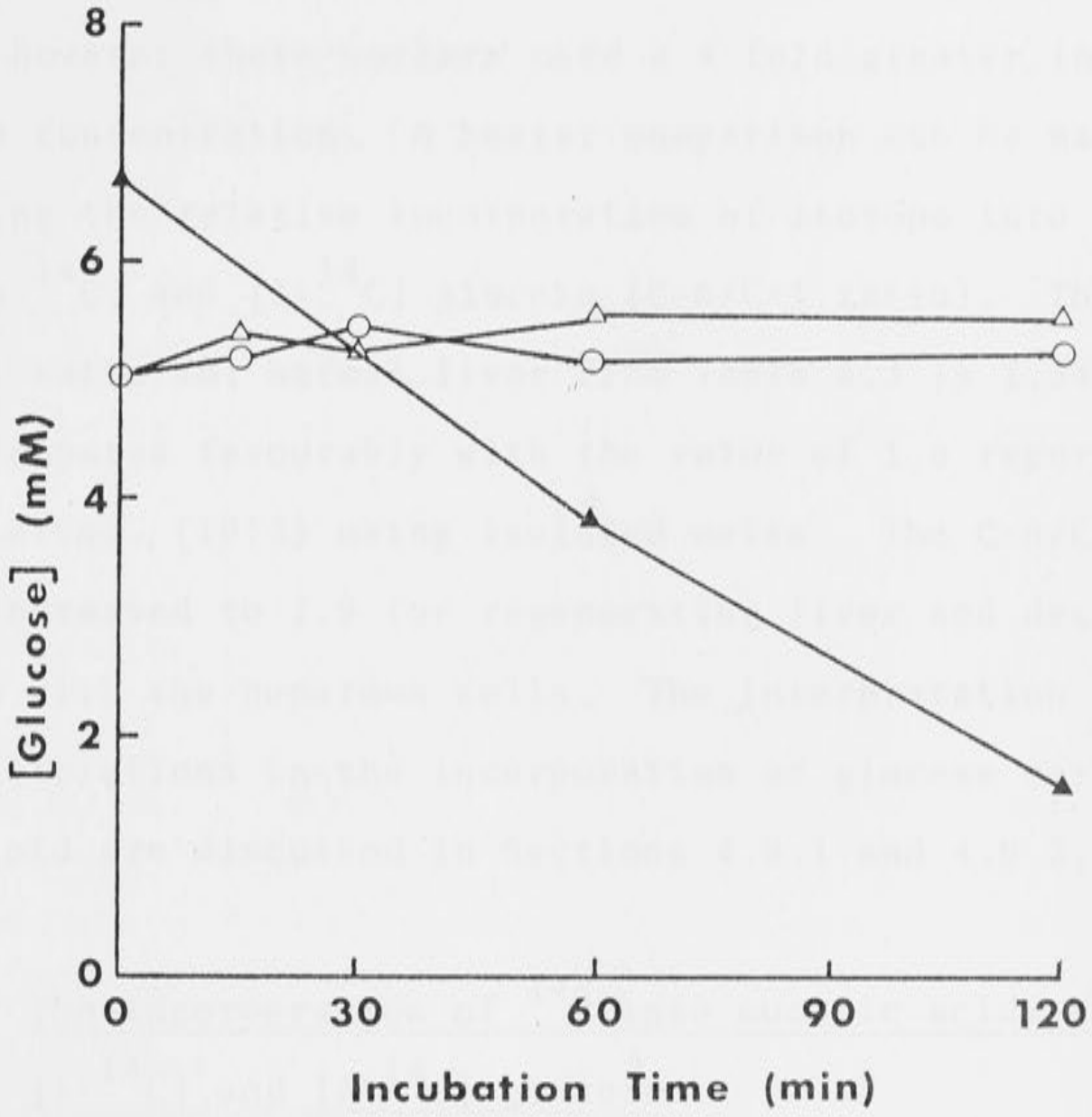



Fig. 4.3: Changes in the glucose concentration of the incubation medium by cells prepared from normal and regenerating liver and hepatoma 5123TC

Isolated cells from normal liver (○), 24h regenerating liver (△) and hepatoma 5123TC (▲) were incubated at 37° for 2h as described in Section 2.12. Aliquots of the cell suspensions were taken at the indicated times, deproteinated using perchloric acid and the glucose concentration determined enzymically (Section 2.8). The results shown represent the average of 3 determinations for each cell type.

incorporation of $^{14}C$ into lipid for normal liver is approximately one third of the values reported by Baquer *et al.* (1973) however these workers used a 4 fold greater initial glucose concentration. A better comparison can be made by examining the relative incorporation of isotope into lipid from [6-$^{14}C$] and [1-$^{14}C$] glucose (C-6/C-1 ratio). The C-6/C-1 ratio for normal liver from Table 4.3 is 1.54 which compares favourably with the value of 1.6 reported by Baquer *et al.* (1973) using isolated cells. The C-6/C-1 ratio increased to 2.9 for regenerating liver and decreased to 0.95 with the hepatoma cells. The interpretation of these alterations in the incorporation of glucose carbon into lipid are discussed in Sections 4.9.1 and 4.9.2.

## 4.6 The incorporation of $^{14}C$ into nucleic acids from [1-$^{14}C$] and [6-$^{14}C$] glucose

The synthesis of RNA and DNA from glucose carbon was measured by following the incorporation of $^{14}C$ into these cell components following the metabolism of [1-$^{14}C$] and [6-$^{14}C$] glucose. The incubation conditions were as described for the lipid studies and the RNA and DNA fractions were isolated as described in Section 2.12. The results are given in Table 4.4. The data show that there is increased incorporation of $^{14}C$ (relative to normal liver) into RNA and DNA by the 5123TC hepatoma cells and to a lesser extent by cells from regenerating liver. It is of interest that the C-6/C-1 ratio in the nucleic acids is approximately 1.3 in all tissues. The significance of this ratio in nucleic acids is discussed in a later section (4.9.2).

Table 4.3: Incorporation of $^{14}C$ into lipid following the metabolism of [1-$^{14}C$] and [6-$^{14}C$] glucose by isolated cells from normal and 24h regenerating liver and hepatoma 5123TC

| Cell | [1-$^{14}C$] Glucose | [6-$^{14}C$] Glucose | C-6/C-1 |
|---|---|---|---|
| Normal Liver | 0.35±0.04 | 0.54±0.10 | 1.54 |
| 24h Regenerating Liver | 0.20±0.02 | 0.58±0.03 | 2.90 |
| Hepatoma 5123TC | 1.40±0.17 | 1.33±0.16 | 0.95 |

Isolated cells from normal liver, 24h regenerating liver and hepatoma 5123TC were incubated with 5mM, [1-$^{14}C$] or [6-$^{14}C$] glucose as described in the legend to Table 4.2 except that 5ml of cell suspension was used and 2.5 μCi of $^{14}C$-labelled glucose was added. The incubations were for 2h and were terminated by the addition of 5ml of 10% (w/v) trichloroacetic acid. Isolation of the lipid fraction is detailed in Section 2.12. The results are expressed as μmoles of glucose carbon per gram wet weight of cells per h and are the mean±SEM of 5 determinations.

Table 4.4: Incorporation of $^{14}C$ into nucleic acids following the metabolism of [1-$^{14}C$] and [6-$^{14}C$] glucose by isolated cells from normal and 24h regenerating liver and hepatoma 5123TC

| Cell | RNA | | |
|---|---|---|---|
| | [1-$^{14}C$] Glucose | [6-$^{14}C$] Glucose | C-6/C-1 |
| Normal Liver | 0.10±0.01 | 0.13±0.02 | 1.30 |
| 24h Regenerating Liver | 0.16±0.02 | 0.20±0.04 | 1.25 |
| Hepatoma 5123TC | 0.57±0.07 | 0.77±0.11 | 1.35 |

| Cell | DNA | | |
|---|---|---|---|
| | [1-$^{14}C$] Glucose | [6-$^{14}C$] Glucose | C-6/C-1 |
| Normal Liver | 0.07±0.01 | 0.09±0.02 | 1.29 |
| 24h Regenerating Liver | 0.09±0.02 | 0.11±0.02 | 1.22 |
| Hepatoma 5123TC | 0.09±0.02 | 0.12±0.03 | 1.33 |

Isolated cells from normal and 24h regenerating liver and hepatoma 5123TC were incubated with [1-$^{14}C$] and [6-$^{14}C$] glucose as described in the legend to Table 4.3. The isolation of RNA and DNA fractions are described in Section 2.12. The results are expressed as µmole of glucose carbon per g wet weight of cells per h and are the mean±SEM of 5 determinations.

4.7 The incorporation of $^{14}C$ into protein from [1-$^{14}C$] and [6-$^{14}C$] glucose

The data of Table 4.5 show the rate of incorporation of $^{14}C$ from specifically labelled glucose into protein. These results show that relative to normal liver there is an increased incorporation of $^{14}C$ into the protein of hepatoma 5123TC cells but a decreased incorporation into protein in 24h regenerating liver cells. This latter result implies that protein synthesis from glucose carbon in regenerating liver cells is not stimulated at 24h at least in these incubation conditions.

4.8 The relative distribution of $^{14}C$ in cellular components and $CO_2$ following the metabolism of [1-$^{14}C$] and [6-$^{14}C$] glucose

The data of Tables 4.2 to 4.5 have been combined to illustrate the percentage recovery of $^{14}C$-isotope in each of the products of glucose metabolism examined and is shown in Table 4.6. The significant features revealed by this analysis of the data are: (1) the very low % of glucose recovered as $^{14}CO_2$ following the metabolism of [6-$^{14}C$] glucose by hepatoma 5123TC; (2) the increased relative $^{14}C$ incorporation into RNA and DNA by 5123TC hepatoma cells and regenerating liver cells; (3) the increased percentage of isotope incorporated into lipid and protein by hepatoma cells relative to normal liver and (4) the decreased incorporation of isotope into protein by regenerating liver. A detailed analysis of the significance of these changes in the various tissues is presented in the following section.

Table 4.5: Incorporation of $^{14}C$ into protein following the metabolism of [1-$^{14}C$] and [6-$^{14}C$] glucose by isolated cells from normal and 24h regenerating liver and hepatoma 5123TC

| Cell | [1-$^{14}C$] Glucose | [6-$^{14}C$] Glucose | C-6/C-1 |
|---|---|---|---|
| Normal Liver | 0.16±0.02 | 0.24±0.07 | 1.50 |
| 24h Regenerating Liver | 0.12±0.02 | 0.13±0.01 | 1.08 |
| Hepatoma 5123TC | 0.30±0.04 | 0.36±0.05 | 1.20 |

Isolated cells from normal and 24h regenerating liver and hepatoma 5123TC were incubated with [1-$^{14}C$] and [6-$^{14}C$] glucose as described in the legend to Table 4.3. The isolation of the protein fraction of cells is described in Section 2.12. The results are expressed as μmole of glucose carbon per gram wet weight of cells per h and are the mean±SEM of 5 determinations.

Table 4.6: The relative distribution of $^{14}C$ into cellular components and $CO_2$ following the metabolism of [1-$^{14}C$] and [6-$^{14}C$] glucose by isolated cells from normal and 24h regenerating liver and hepatoma 5123TC

| Product | Percentage of Recovered $^{14}C$ | | | | | |
|---|---|---|---|---|---|---|
| | Normal Liver | | 24h Regen. Liver | | Hepatoma 5123TC | |
| | [1-$^{14}C$] Glucose | [6-$^{14}C$] Glucose | [1-$^{14}C$] Glucose | [6-$^{14}C$] Glucose | [1-$^{14}C$] Glucose | [6-$^{14}C$] Glucose |
| $CO_2$ | 64 | 39 | 72 | 39 | 47 | 7 |
| Lipid | 19 | 33 | 10 | 35 | 32 | 48 |
| RNA | 5 | 8 | 8 | 12 | 13 | 28 |
| DNA | 4 | 5 | 5 | 7 | 2 | 4 |
| Protein | 8 | 15 | 6 | 8 | 7 | 13 |
| Total $^{14}C$ incorporated (µmole/g/h) | 1.89 | 1.65 | 2.00 | 1.68 | 4.44 | 2.78 |

The data of Tables 4.2 to 4.5 have been combined and the percentage of the isotope recovered in each cell product calculated. The total rate of glucose incorporation into products was determined by adding the rates of $^{14}C$ incorporation reported in Tables 4.2 to 4.5.

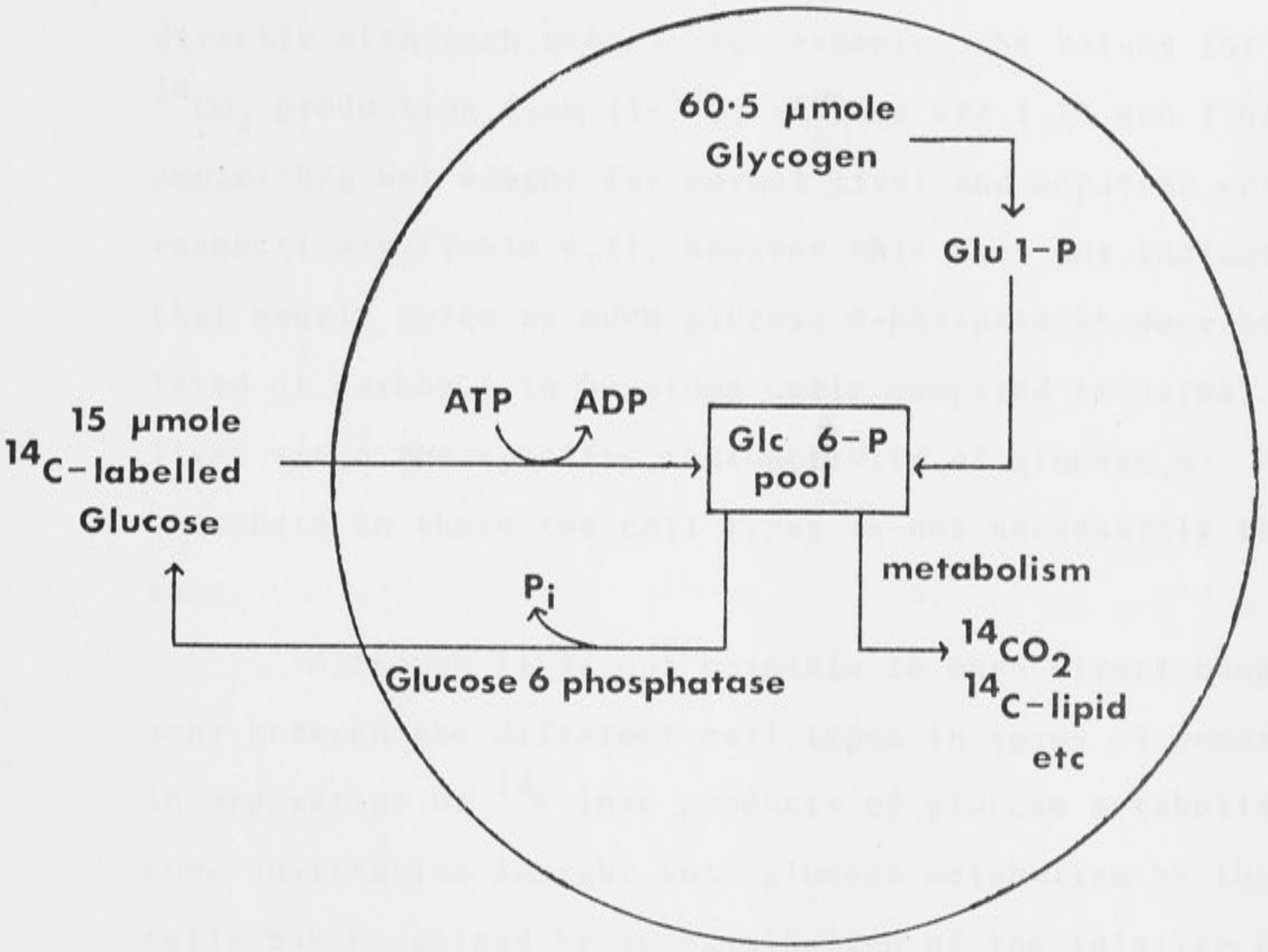
## 4.9 Discussion

The interpretation of these $^{14}C$-labelled glucose studies is exceedingly complex. The experiments described above were designed to detect differences in the direction of flow of glucose carbon in the various cell types and to establish whether or not there is notable variation in the flux of glucose 6-phosphate through the pentose phosphate pathway. To make these comparisons, the incubation conditions and the calculations of results have been standardized, however the different nature of the cells used in this study limit the interpretation of the results. In particular, the glycogen content of the various cells makes direct comparison of the amount of $^{14}C$-isotope in products impossible. The data of Fig. 4.3 shows that there is no net utilization of glucose during the experimental period by hepatocytes from either normal or 24h regenerating liver although there is significant net utilization by hepatoma cells. The failure of hepatocytes to utilize glucose from the medium is presumably a result of glycogenolysis under the incubation conditions adopted. Glycogenolysis is an option which is not available to hepatoma cells since they have only very low levels of glycogen. This assumption is supported by the data of Katz *et al.* (1975) who demonstrated a net rate of glycogen disappearance of 24 µmol/h/100mg of protein in the absence of any changes in glucose concentration when cells isolated from livers of rats fed *ad libitum* were incubated with glucose at a concentration of 20mM. The influence of a large pool of glycogen in hepatocytes on the amount of $^{14}C$ incorporated into products of glucose 6-phosphate metabolism is

illustrated in Scheme 4.1. Hepatocytes from normal liver contain a large pool of unlabelled glucose 6-phosphate precursor, i.e. glycogen. In the incubation conditions used in these experiments, the unlabelled pool greatly exceeds the pool of $^{14}$C-glucose in the incubation media and the resulting specific radioactivity of the glucose 6-phosphate will clearly depend on the relative rates of glycogenolysis and phosphorylation of glucose. The amount of $^{14}$C-isotope in reaction products of glucose 6-phosphate, i.e. $CO_2$, lipid etc., will also be dependent on these factors. Thus comparisons of the amount of $^{14}$C-isotope in normal and regenerating liver may be misleading since the size of the glycogen pool is reduced in regenerating liver and the balance of the two glucose phosphorylating enzymes (hexokinase and glucokinase) is also altered in this tissue (Section 1.4.2). Comparisons with hepatoma cells are obviously influenced by glycogenolysis since the hepatoma cells contain very little glycogen and do show net utilization of glucose from the incubation medium. It should be noted that use of glycogen-depleted cells (i.e. from starved rats) cannot overcome this problem since in this case the cells are very actively synthesizing glucose and the incorporation of $^{14}$C-isotope into metabolic intermediates under similar incubation conditions is much reduced (Katz *et al.*, 1975).

It is emphasized that although the results presented in this chapter are expressed as µmoles of glucose carbon incorporated into products/h/g wet weight, these calculations were based on the specific radioactivity of the substrate glucose in the incubation and may not reflect

Scheme 4.1: The influence of glycogenolysis and glucose phosphorylation on the specific radioactivity of glucose 6-phosphate




The scheme shows the effect of glycogenolysis on the dilution of the specific radioactivity of glucose 6-phosphate under the incubation conditions used in these experiments. The example used is the 3ml incubation of cells for $^{14}CO_2$ studies which contained 15µmole of $^{14}C$-labelled glucose and approx. 250 mg wet weight of cells. The 60.5µmole of glucose equivalent in glycogen was calculated based on a glycogen content in normal liver of 242µmoles/g fresh weight (Knox, 1972). It can be seen that the pool of unlabelled glucose 6-phosphate precursor is 4 times as large as the $^{14}C$-labelled pool. The specific radioactivity of the glucose 6-phosphate must then be a function of the relative rates of phosphorylation and of glycogenolysis. In the case of 24h regenerating liver the glycogen pool size is approx. 28µmoles.

the specific radioactivity of glucose 6-phosphate in the different cells. Thus the values cannot be compared directly with each other. For example, the values for $^{14}CO_2$ production from [1-$^{14}$C] glucose are 1.21 and 2.08 μmoles/h/g wet weight for normal liver and hepatoma cells respectively (Table 4.2), however this does not indicate that nearly twice as much glucose 6-phospate is decarboxylated at carbon 1 in hepatoma cells compared to normal liver since the specific radioactivity of glucose 6-phosphate in these two cell types is not necessarily the same.

Although it is not possible to make direct comparisons between the different cell types in terms of rates of incorporation of $^{14}$C into products of glucose metabolism, some qualitative insight into glucose metabolism by these cells may be gained by an examination of the relative distribution of $^{14}$C-isotope in terms of percentages (Table 4.6) and by comparing the relative incorporation of isotope from [1-$^{14}$C] and [6-$^{14}$C] glucose. These analyses are presented in the following sections.

### 4.9.1 Analysis of the distribution of $^{14}$C in various cell components and $CO_2$.

The data of Table 4.6 indicates that the greatest proportion of $^{14}$C-isotope from both [1-$^{14}$C] and [6-$^{14}$C] glucose is recovered in $CO_2$ and lipid following metabolism of these substrates by isolated hepatocytes and there is very little difference in this pattern for regenerating liver cells. The metabolism by the tumour cells, however, is significantly different with only 9% of the recovered

isotope found in $CO_2$ when [6-$^{14}$C] glucose is the substrate. The notable decrease in the $^{14}CO_2$ production from [6-$^{14}$C] glucose in hepatoma 5123TC is accompanied by a marked increase in the proportion of isotope incorporated into lipid, RNA and DNA. This observation implies that metabolism of glucose by the hepatoma cells is characterised by a decreased activity of the tricarboyxlic acid cycle and increased synthesis of lipid and nucleic acid relative to the flow of glucose carbon in normal liver. The data for 24h regenerating liver also demonstrates an increased relative incorporation of $^{14}$C-isotope into RNA and DNA but not into lipid in spite of the observation of Gove and Hems (1978) that *de novo* fatty acid synthesis is increased in this tissue.

It is of interest to compare the results of Table 4.6 with those of Crockett and Leslie (1963) who studied human-foetal-liver cells (Strain HLM). These workers grew the foetal liver cells in culture for 48h with [1-$^{14}$C] and [6-$^{14}$C] glucose and fractionated the cells to measure the incorporation of $^{14}$C-labelled glucose carbon into nucleic acids, lipids, proteins and acid soluble compounds as well as $CO_2$. When their data is re-examined in terms of the cell components measured in this study the distribution of isotope is as follows:

| | % of $^{14}$C-isotope from: | |
|---|---|---|
| Component | [1-$^{14}$C] glucose | [6-$^{14}$C] glucose |
| $CO_2$ | 64 | 30 |
| Lipid | 13 | 32 |
| RNA | 10 | 18 |
| DNA | 3 | 5 |
| Protein | 10 | 15 |

Comparison of this data with that given in Table 4.6 shows general agreement with the pattern of $^{14}C$-isotope distribution found in the cells studied here. The alterations from normal liver are a decreased incorporation into lipid from [1-$^{14}C$] glucose and increased incorporation into RNA from both [1-$^{14}C$] and [6-$^{14}C$] glucose. Thus the qualitative pattern is similar to that seen in regenerating liver. However, quantitatively the incorporation of isotope into RNA more closely resembles the hepatoma 5123TC, and the decreased incorporation of $^{14}C$ into $CO_2$ from [6-$^{14}C$] glucose suggests a less active tricarboxylic acid cycle in these cells. It is of note that these cells in culture do show a net utilization of glucose, i.e. glucose is a good substrate for foetal liver cells.

4.9.2 Differences in recovery of $^{14}C$ following the metabolism of [1-$^{14}C$] and [6-$^{14}C$] glucose

The incorporation of $^{14}C$-isotope into the various cellular components and $CO_2$ following the metabolism of [1-$^{14}C$] and [6-$^{14}C$] glucose by cells (Tables 4.2 to 4.5) can be analyzed in terms of the relative activity of the pentose phosphate pathway or the pentose cycle since the oxidative segment of the pathway yields $^{14}CO_2$ from [1-$^{14}C$] glucose 6-phosphate but not from [6-$^{14}C$] glucose 6-phosphate. It is emphasized that values for the quantitative participation of the pentose cycle relative to other pathways of glucose metabolism cannot be accurately determined from the data presented in this chapter (see Sections 1.3 and 4.9.3), however, some qualitative comparisons between tissues can be made.

The yield of $^{14}CO_2$ from [1-$^{14}$C] glucose exceeds that from [6-$^{14}$C] glucose in all three cell types examined (Fig. 4.1). This observation is taken to show that an active oxidative segment of the pentose pathway operates in all of these tissues. The ratio of $^{14}CO_2$ production from [1-$^{14}$C] glucose relative to that from [6-$^{14}$C] glucose (i.e. the C-1/C-6 ratio) is an indicator of the relative activity of the oxidative segment of the pentose pathway and the tricarboxylic acid cycle. The ratios are 1.86 for normal hepatocytes, 2.17 for regenerating liver and 10.4 for hepatoma 5123TC. These ratios indicate that the hepatoma cells show a greater degree of oxidative decarboxylation *via* the oxidative segment of the pentose pathway relative to the tricarboxylic acid cycle than either normal or regenerating liver. It is emphasized that differences in the C-1/C-6 ratios in $CO_2$ depends largely (but not exclusively) on the oxidative segment of the pentose pathway rather than the complete pentose cycle, but is not an absolute indication of actual flux increase through the pentose pathway since the ratio is also a reflection of the activity of the tricarboxylic acid cycle.

The incorporation of $^{14}$C-isotope into lipid from [1-$^{14}$C] and [6-$^{14}$C] glucose may also be interpreted in terms of the activity of the pentose phosphate pathway. $^{14}$C-labelled triose phosphate will be formed from [6-$^{14}$C] glucose by both the glycolytic and the pentose phosphate pathways whereas $^{14}$C-labelled triose phosphate will be formed from [1-$^{14}$C] glucose only *via* the glycolytic pathway. In the presence of an active pentose pathway, the incorporation of $^{14}$C-isotope will be greater from [6-$^{14}$C] glucose

than [1-$^{14}$C] glucose, i.e. the C-6/C-1 ratio in lipid will be greater than 1 (note, in terms of the L-type pathway this is only valid if there is equilibration of triose phosphate pools, see Section 1.3.2). Thus the activity of the pentose pathway relative to the glycolytic pathway may be compared for each cell type by examining the C-6/C-1 ratio in lipid. The ratios are 1.54 for normal liver, 2.86 for 24h regenerating liver, and 0.95 for hepatoma 5123TC. These results imply a more active pentose pathway in regenerating liver than normal liver and no pentose pathway activity for hepatoma 5123TC. The C-6/C-1 ratio for hepatoma 5123TC appears to contradict the implications of the C-1/C-6 ratio in $CO_2$ discussed above. However, it must be remembered that the C-1/C-6 ratio is a qualitative assessment of the oxidative segment of the pentose pathway relative to the tricarboxylic acid cycle whereas the C-6/C-1 ratio in lipid is an indication of the relative rates of net triose-phosphate formation *via* the glycolytic and pentose phosphate pathways. Thus the apparent contradiction is solved if it is assumed that the activity of the tricarboxylic acid is low and the activity of the glycolytic pathway is high in hepatoma 5123TC. An alternative explanation for the failure to detect any difference in the amount of $^{14}$C-isotope incorporated into lipid of hepatoma cells from [1-$^{14}$C] and [6-$^{14}$C] glucose is the influence of $^{14}$C-isotope exchange reactions. If the rate of these exchange reactions is rapid relative to the flux of carbon through the pentose phosphate pathway, then $^{14}$C-labelled pentose 5-phosphate will be formed from [1-$^{14}$C] glucose and the dilution effect on the specific radioactivity of

triose-phosphates would not be observed. Examples of such exchange reactions are discussed below.

The incorporation of $^{14}C$-isotope into nucleic acids from [1-$^{14}C$] and [6-$^{14}C$] glucose is a useful indicator of the contribution of the oxidative segment of the pentose pathway to pentose 5-phosphate formation relative to the activity of $^{14}C$-isotope exchange reactions and/or the reversal of the non-oxidative segment of the cycle. When [1-$^{14}C$] glucose is the substrate, the pentose 5-phosphate formed by the oxidative segment of the pentose pathway will be unlabelled whereas $^{14}C$-labelled pentose 5-phosphate is formed from [6-$^{14}C$] glucose. The data of Table 4.4 show that the C-6/C-1 ratio for RNA and DNA is approximately 1.3 in all cells. This data suggests that the pentose 5-phosphate incorporated into nucleic acid is not formed primarily by the oxidative segment of the pentose pathway and is consistent with similar studies by Gumaa and McLean (1969) using Krebs ascites cells, Kit *et al*. (1957) using lymphatic cells and tumours, Hiatt (1957) using HeLa cells, Schneider and Potter (1957) with regenerating liver, and Schmitz *et al*. (1954) using the Flexner-Jobling carcinoma. These workers interpreted their results as indicating that a significant proportion of the ribose 5-phosphate moiety of nucleic acid was formed by reversal of the non-oxidative segment of the pentose pathway. However, this interpretation may be misleading since measurements of the incorporation of $^{14}C$-isotope into products often does not measure flux of carbon, but rather exchange of isotope. There is good evidence that *in vitro* the flow of carbon is from pentose 5-phosphate to hexose 6-phosphate. Williams and

Blackmore (unpublished results) incubated $^{14}$C-labelled glucose 6-phosphate and fructose 1,6 bisphosphate (as a source of triose phosphate) with a rat liver enzyme preparation and showed only 4 to 6% of the $^{14}$C-isotope associated with pentose 5-phosphate. These results clearly demonstrate that, at least *in vitro*, the contribution of the non-oxidative segment of the pentose phosphate pathway to *net* pentose 5-phosphate formation is very small. The alternate explanation for the very low C-6/C-1 ratios found in nucleic acids is the operation of $^{14}$C-isotope exchange reactions in which there is no net flux of carbon. Examples of such exchange reactions which would result in $^{14}$C incorporation into pentose 5-phosphate from [1-$^{14}$C] glucose or hexose 6-phosphate are shown in equations 4.1 to 4.3 all catalyzed by transketolase.

$$[1\text{-}^{14}C]\ \text{Fru 6-P} + \text{Xlu 5-P} \rightleftharpoons [1\text{-}^{14}C]\ \text{Xlu 5-P} + \text{Fru 6-P} \quad (4.1)$$

$$[3\text{-}^{14}C]\ \text{Gra-P} + \text{Xlu 5-P} \rightleftharpoons [5\text{-}^{14}C]\ \text{Xlu 5-P} + \text{Gra-P} \quad (4.2)$$

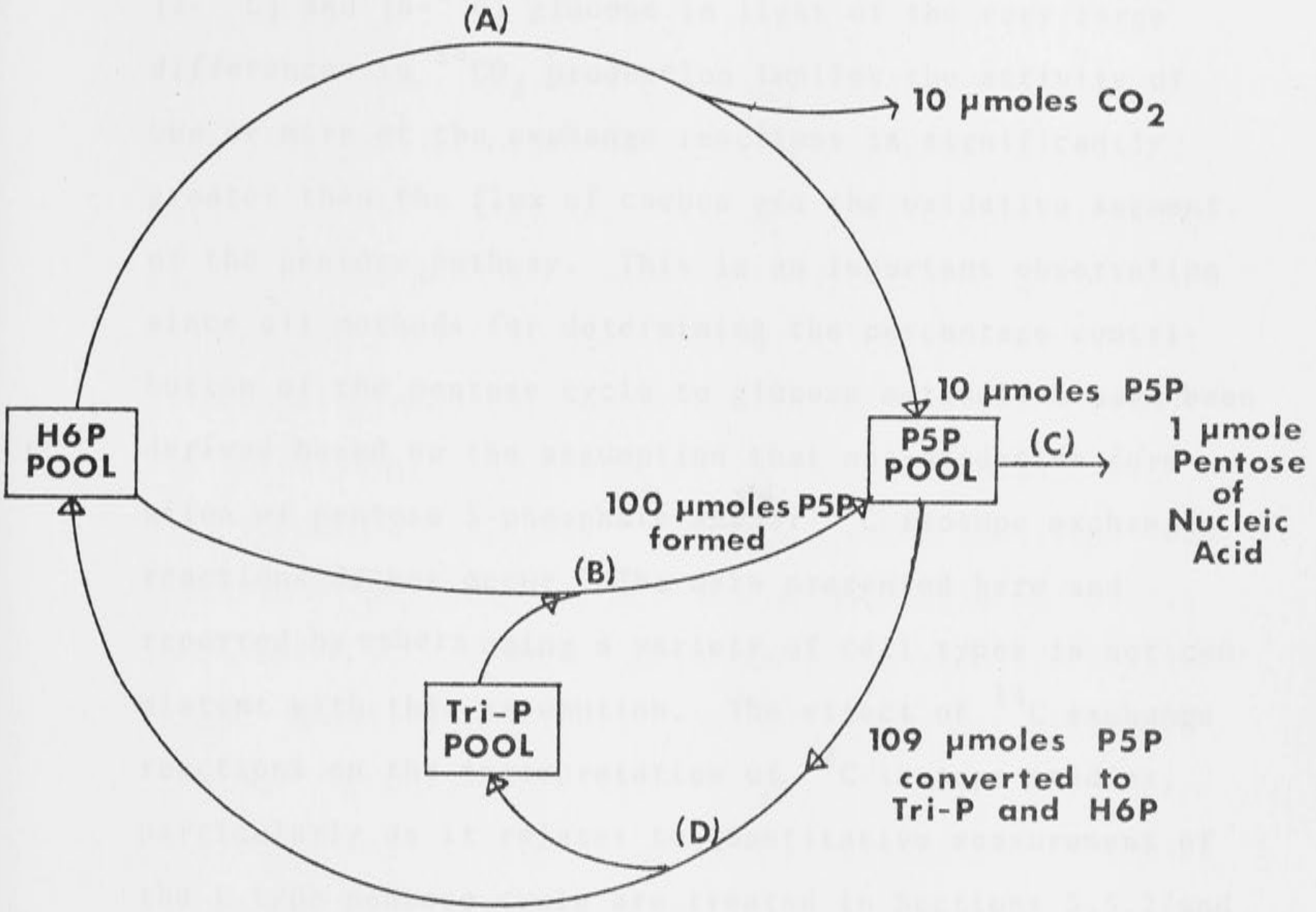
$$[1\text{-}^{14}C]\ \text{Seh 7-P} + \text{Xlu 5-P} \rightleftharpoons [1\text{-}^{14}C]\ \text{Xlu 5-P} + \text{Seh 7-P} \quad (4.3)$$

The [3-$^{14}$C] glyceraldehyde 3-phosphate of equation 4.2 is formed from [1-$^{14}$C] fructose 6-phosphate *via* the glycolytic pathway (see Reactions A, B and C of Scheme 5.II). The [1-$^{14}$C] heptulose 7-phosphate of equation 4.3 may be formed from the transaldolase catalyzed reaction between [1-$^{14}$C] fructose 6-phosphate and erythrose 4-phosphate which yields [1-$^{14}$C] heptulose 7-phosphate and glyceraldehyde 3-phosphate.

Although the *net* synthesis of pentose 5-phosphate *via* reversibility of the non-oxidative segment of the pentose pathway (either the F-type or the L-type) is low, the action of this series of reactions may be considered to

constitute a cycle through which a significant amount of $^{14}C$-isotope may be incorporated into pentose 5-phosphate in the absence of net synthesis. The operation of this exchange cycle is best illustrated in Scheme 4.2 which shows hypothetical rates of the flow of carbon through the oxidative and non-oxidative segments of the pentose pathway and into nucleic acids. The Scheme shows a net flux of glucose carbon in the forward direction of the pentose cycle of 9, however the pentose 5-phosphate pool will contain a significant amount of $^{14}C$-isotope even when [1-$^{14}C$] glucose is substrate as a consequence of the rapid rate of the $^{14}C$ exchange cycle (Reactions B and D) relative to the net flux of carbon. The amount of $^{14}C$-isotope in the nucleic acids will reflect the amount of isotope in the pool of pentose 5-phosphate. This amount will be determined by the inflow of isotope from the exchange cycle plus the inflow from the oxidative segment of the pentose pathway when [6-$^{14}C$] glucose is substrate but will be equal to the inflow *via* the exchange cycle only when [1-$^{14}C$] glucose is used. In the example illustrated in the Scheme 4.2 it would be difficult to detect any difference in the amount of isotope from [1-$^{14}C$] and [6-$^{14}C$] glucose. The important distinction is that the $^{14}C$ exchange reactions do not represent net carbon flux, and therefore it is in error to conclude that a significant proportion of pentose 5-phosphate has been formed *via* the non-oxidative segment of the pentose cycle, although it may be true that a significant proportion of $^{14}C$-isotope is incorporated into pentose 5-phosphate *via* $^{14}C$-exchange reactions. The observation that there is a very little difference between the

Scheme 4.2: The role of $^{14}C$ exchange in the labelling of pentose 5-P




The scheme shows the conversion of 10µmoles of glucose 6-phosphate to pentose 5-phosphate *via* the oxidative segment of the pentose pathway (A). A further 100µmoles of pentose 5-phosphate is formed by non-oxidative reactions (B). One µmole of the pentose 5-phosphate is utilized for nucleic acid synthesis (C) and 109µmoles of pentose 5-phosphate is metabolized *via* the non-oxidative segment of the pentose pathway to yield hexose 6-P and triose-P (D). The net flow of carbon is from pentose 5-P to hexose 6-P, however with $^{14}C$-labelled glucose the $^{14}C$ incorporation into pentose and therefore nucleic acids will be more influenced by the non-oxidative pathway than the oxidative pathway. The concerted action of the reactions B and D represent the $^{14}C$-exchange cycle.

incorporation of $^{14}C$-isotope into nucleic acids from [1-$^{14}C$] and [6-$^{14}C$] glucose in light of the very large differences in $^{14}CO_2$ production implies the activity of one or more of the exchange reactions is significantly greater than the flux of carbon *via* the oxidative segment of the pentose pathway. This is an important observation since all methods for determining the percentage contribution of the pentose cycle to glucose metabolism have been derived based on the assumption that non-oxidative formation of pentose 5-phosphate and/or $^{14}C$-isotope exchange reactions do not occur. The data presented here and reported by others using a variety of cell types is not consistent with this assumption. The effect of $^{14}C$ exchange reactions on the interpretation of $^{14}C$-isotope studies, particularly as it relates to quantitative measurement of the L-type pentose cycle are treated in Sections 5.5.2 and 8.2.

It is of note that the operation of the $^{14}C$ exchange cycle will also influence the specific radioactivity of the pentose 5-phosphate pool and thus the pool of triose-phosphate and of lipid. For example, when [1-$^{14}C$] glucose is the substrate and no exchange operates, the pentose 5-phosphate acting as substrate for triose-phosphate formation will contain no isotope. However, when $^{14}C$-isotope exchange acts much more rapidly than flux, the specific radioactivity of the pentose 5-phosphate pool will be high and the dilution effect much less than in its absence. This effect of $^{14}C$-isotope exchange may explain in part the very low C-6/C-1 ratio found in the lipid of hepatoma cells.

4.9.3 Quantitative estimates of the pentose phosphate cycle

As described in a previous section (1.3) methods have been derived based on a simplified model of glucose metabolism for estimating the quantitative participation of the F-type pentose cycle. Although the results presented here indicate that estimates based on this model and its limiting assumptions are not valid in liver tissues, the calculations are presented to demonstrate the difficulties in such an analysis. The calculation involving $^{14}CO_2$ specific yields requires a measurement of glucose utilization which as discussed above cannot be made for hepatocytes. However, a value of 5.0 µmoles/h/g wet weight is used for the purpose of this exercise based on the "apparent" utilization measurements of Katz *et al.* (1975) and Baquer *et al.* (1973). The rate of glucose utilization for hepatoma cells of 30 µmoles/h/g which was determined experimentally (Fig. 4.2 and Section 4.4) was used to calculate the percentage pentose cycle contribution for these cells and represents real rather than "apparent" utilization. The values for percentage pentose cycle based on $^{14}CO_2$ specific yields using equation 1.1 are 5% for normal liver, 7% for regenerating liver and 2% for hepatoma 5123TC. The low value for the 5123TC is a consequence of the small percentage of utilized glucose converted to $CO_2$ and is not a reflection of the C-1/C-6 ratio whereas the low values for normal and regenerating liver reflect the low C-1/C-6 ratios. This calculation for the hepatoma cells indicates that the majority of glucose utilized by these cells is *via* glycolysis. This conclusion is

supported by the observation that only 4.4 µmoles of glucose carbon can be accounted for in $CO_2$ and other cell products analyzed (Table 4.6) whereas 30 µmole is utilized. Thus 85% of glucose may be considered to be converted to acid soluble products and the majority of this is probably lactate. The values of percentage pentose cycle calculated from equation 1.2 based on the incorporation of $^{14}C$ into lipid are 15% for normal liver, 38% for regenerating liver and 0% for hepatoma 5123TC. Obviously the results of these calculations vary greatly and illustrate the fact that application of expressions derived for the F-type pentose cycle to liver tissue does not give valid or consistent estimates of the contribution of the pentose pathway to glucose metabolism.

4.9.4 Conclusions

The fate of $^{14}C$-isotope following the metabolism of [1-$^{14}C$] and [6-$^{14}C$] glucose by isolated cells from normal and regenerating liver and hepatoma 5123TC indicates that there is a marked change in the metabolism of this substrate by neoplastic hepatic tissue relative to normal liver. The results indicate that there is a decreased activity of the tricarboxylic acid cycle, an increase in glycolysis, and redirection of glucose carbon into protein, lipid and nucleic acids by the hepatoma. The regenerating liver cells also demonstrate an increase in the incorporation of isotope from glucose into RNA and DNA but a decreased incorporation into protein.

The quantitative role of the pentose phosphate pathway in these 'growth' tissues remains unclear. Direct

comparisons of the actual flux of glucose into $CO_2$ and other cell components are not possible as hepatocytes do not utilize glucose from the incubation media and results in uncertainty regarding the specific radioactivity of glucose 6-phosphate in each of the cell types. Qualitative comparisons are also difficult to make since the C-1/C-6 ratios in $CO_2$ could reflect alterations in either or both the tricarboxylic acid cycle and the oxidative segment of the pentose pathway and C-6/C-1 ratios in lipid are dependent on the glycolytic rate and the equilibration of pools of triose phosphate as well as the activity of the pentose pathway. The assessment of the data presented here is further complicated by the apparent operation of $^{14}C$-isotope exchange reactions as indicated by the C-6/C-1 ratio in nucleic acids. Perhaps the most significant observation to be made from the results presented in this chapter is the inadequacy of presently available methods using $^{14}C$-labelled substrates to determine the relative quantitative role of the pentose cycle in various tissues, particularly hepatic tissues. A critical evaluation of the results presented here highlights the need for an alternative approach to examining the metabolism of glucose by hepatic tissues particularly as it relates to the pentose phosphate pathway. Ideally such an approach must attempt to deal with the following problems which are evident from the above discussion: (1) it must attempt to distinguish between and/or measure independently the flow of carbon through the F-type and L-type pentose cycles; (2) the measurements should be independent of glucose utilization; (3) the method should be independent of or capable of

distinguishing between flux of glucose carbon and $^{14}C$-isotope exchange, either from reversibility of the non-oxidative segment of the pentose cycle or by the exchange reactions discussed above; (4) the measurement should yield a value for the percentage contribution of the pentose cycle (F-type or L-type) which is relative to total glucose metabolism including metabolism by non-triose phosphate yielding pathways such as glycogen and nucleic acid synthesis. It was considered that development of an experimental approach which attempted to fulfill these criteria was essential to any further efforts to investigate the role of the pentose phosphate pathway in these tissues. The remainder of this thesis is devoted to the theoretical derivation and application of a new experimental method for estimating the quantitative participation of the L-type pentose cycle in liver tissues.

# CHAPTER 5

## A THEORETICAL METHOD FOR ESTIMATING THE QUANTITATIVE PARTICIPATION OF THE L-TYPE PENTOSE PHOSPHATE CYCLE

### 5.1 Prefatory comment

The purpose of this chapter is to present the derivation of theoretical expressions for estimating the contribution of the L-type pentose cycle to the metabolism of glucose by tissues. As discussed in Chapter 1 (Section 1.3) the best estimates of the F-type pentose cycle are based on the redistribution of the carbon atoms of hexose by their ordered passage through the reactions of that pathway. This approach allows for an estimate of the quantitative contribution of the pentose cycle relative to total glucose metabolism (including those pathways which do not yield triose-phosphates) and is independent of the rate of glucose utilization. The approach discussed here is a modification of that originally used by Wood and Katz (1958) and Katz and Wood (1960) and is based on the unique carbon atom distributions resulting from the reactions of the L-type pathway. The carbon redistributions *via* the L-type pathway have been analyzed in two ways. The first is expressed as tabulations of the changes in the specific radioactivity of the various carbon atoms of hexose as they pass through a number of cycles of the pentose pathway. This approach is similar to that of Wood and Katz (1958) and results in plots of percentage pentose cycle as a function of the ratios of the specific radioactivities of specific carbon atoms. This analysis is shown in the

appendix. The alternative approach involves the algebraic derivation of expressions based on procedures described by Landau *et al*. (1964) and Katz and Rognstad (1967). These derivations are detailed in this chapter. The assumptions made in deriving these expressions and the limitations of their application are discussed particularly in relation to the effects of $^{14}C$-isotope exchange reactions.

## 5.2 The distribution of $^{14}C$ from [5-$^{14}C$] glucose by reactions of the L-type pentose phosphate cycle

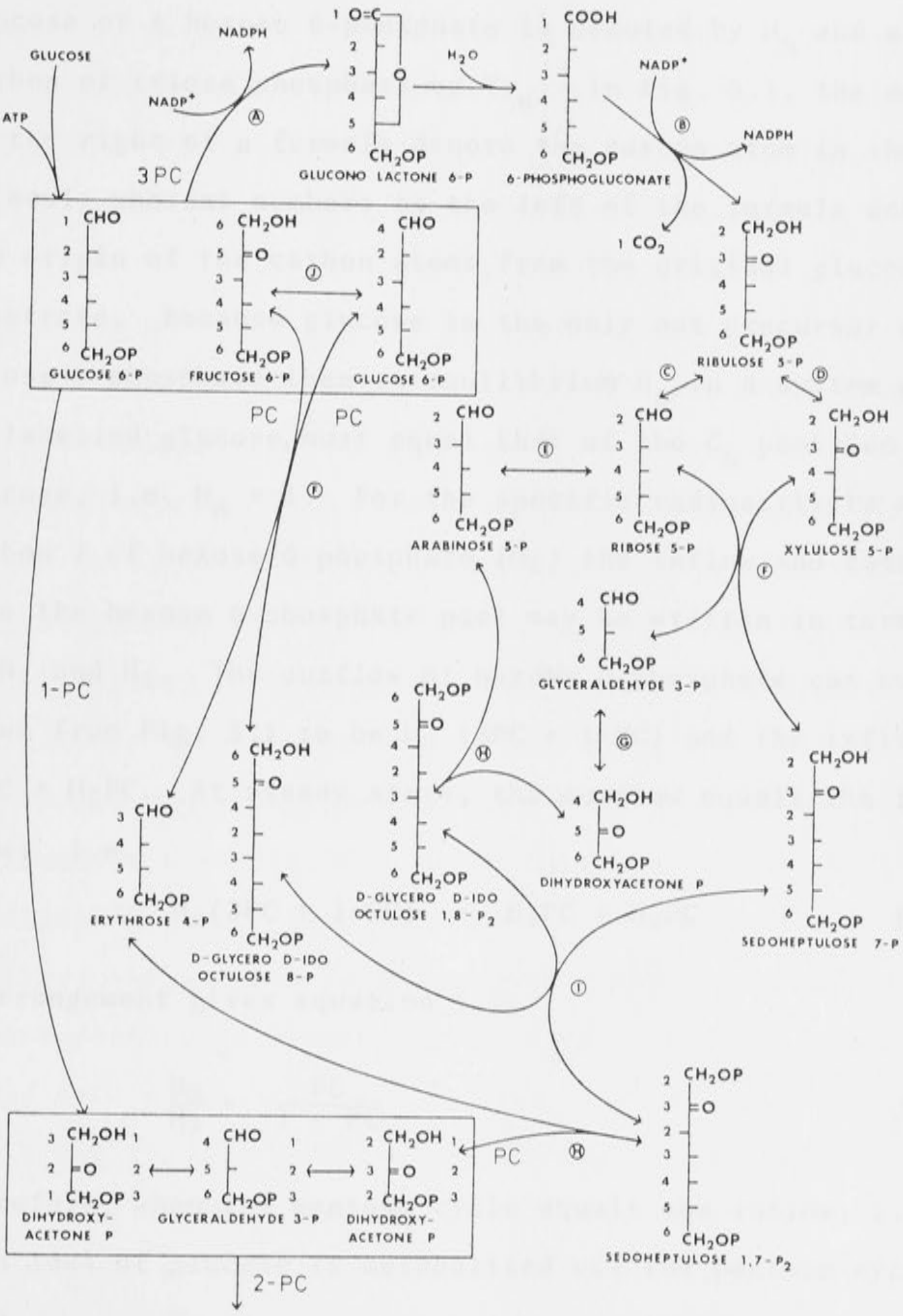
The redistribution of the carbon atoms of glucose by the reactions of the non-oxidative segment of the L-type pentose phosphate cycle is shown in Fig. 5.1. It is significant that formation of hexose 6-phosphate by these reactions leads to the ordered redistribution of carbon atoms 4,5 and 6 of the original glucose molecule into the top three carbons of hexose 6-phosphate. This distribution is unique and distinguishes between the L-type and F-type mechanisms for the non-oxidative segment of the cycle. The estimate is based on the use of [5-$^{14}C$] glucose as substrate and the unique relocation of $^{14}C$ from position 5 into position 2 of glucose 6-phosphate (Fig. 5.1). The metabolism of [5-$^{14}C$] glucose by the test tissue is then followed by the isolation and degradation of the glucose moiety of glucose 6-phosphate (Williams *et al*., 1978a). The distribution of $^{14}C$ in C-2 of glucose 6-phosphate thus serves as a test of the operation of the L-type pentose cycle and as the measure of its relative contribution to total glucose metabolism.

Figure 5.1 presents a simplified model showing glucose metabolism *via* the L-type pentose cycle and the

Fig. 5.1: Redistribution of the carbon atoms of glucose by the L-type pentose phosphate cycle

The sequence of reactions comprising the pentose phosphate cycle in liver as proposed by Williams *et al.* (1978b) is shown. The carbon atoms of the original glucose are indicated to the left of the structural formulae of each of the intermediates and the numbers to the right of the triose phosphates represent the carbon atoms of that molecule. The enzymes involved in the cycle are indicated by the letters and are: (A) glucose 6-phosphate dehydrogenase; (B) 6-phosphogluconate dehydrogenase; (C) ribose 5-phosphate isomerase; (D) ribulose 5-phosphate 3 epimerase; (E) arabinose 5-phosphate epimerase; (F) transketolase; (G) triosephosphate isomerase; (EC 5.3.1.1); (H) aldolase; (EC 4.1.2.13); (I) D-*glycero*-D-*ido*-octulose 1,8 bisphosphate : D-*altro*-heptulose 7-phosphotransferase; (J) glucosephosphate isomerase.

# FIG 5.1


GLUCOSE
ATP
NADPH
NADP+
Ⓐ
3 PC
1 O=C
2
3
4
5
6 CH2OP
GLUCONO LACTONE 6-P
H2O
1 COOH
2
3
4
5
6 CH2OP
6-PHOSPHOGLUCONATE
NADP+
Ⓑ
NADPH
1 CO2
2 CH2OH
3 =O
4
5
6 CH2OP
RIBULOSE 5-P
Ⓒ
Ⓓ
1 CHO
2
3
4
5
6 CH2OP
GLUCOSE 6-P
6 CH2OH
5 =O
3
4
5
6 CH2OP
FRUCTOSE 6-P
Ⓙ
4 CHO
2
3
4
5
6 CH2OP
GLUCOSE 6-P
PC
PC
Ⓕ
2 CHO
3
4
5
6 CH2OP
ARABINOSE 5-P
Ⓔ
2 CHO
3
4
5
6 CH2OP
RIBOSE 5-P
2 CH2OH
3 =O
4
5
6 CH2OP
XYLULOSE 5-P
Ⓕ
4 CHO
5
6 CH2OP
GLYCERALDEHYDE 3-P
Ⓖ
1-PC
6 CH2OP
5 =O
4
2
3
4
5
6 CH2OP
D-GLYCERO D-IDO
OCTULOSE 1,8-P2
Ⓗ
4 CH2OH
5 =O
6 CH2OP
DIHYDROXYACETONE P
2 CH2OH
3 =O
2
3
4
5
6 CH2OP
SEDOHEPTULOSE 7-P
3 CHO
4
5
6 CH2OP
ERYTHROSE 4-P
6 CH2OH
5 =O
4
2
3
4
5
6 CH2OP
D-GLYCERO D-IDO
OCTULOSE 8-P
Ⓘ
2 CH2OP
3 =O
2
3
4
5
6 CH2OP
SEDOHEPTULOSE 1,7-P2
3 CH2OH 1
2 =O
1 CH2OP 3
DIHYDROXY-
ACETONE P
4 CHO 1
5
6 CH2OP 3
GLYCERALDEHYDE 3-P
2 CH2OH 1
3 =O 2
2 CH2OP 3
DIHYDROXY-
ACETONE P
PC
Ⓗ
2-PC

Embden-Meyerhof pathway. The rate of the pentose cycle is designated by PC relative to a net input of substrate glucose. The rate of glucose phosphorylation and the specific radioactivity of substrate glucose have defined values equal to one. The specific radioactivity of any carbon n in glucose or a hexose 6-phosphate is denoted by $H_n$ and any carbon of triose phosphate by $Tr_n$. In Fig. 5.1, the numbers to the right of a formula denote the carbon atom in the molecule whereas numbers to the left of the formula denote the origin of the carbon atoms from the original glucose substrate. Because glucose is the only net precursor of hexose 6-phosphate then at equilibrium $H_n$, in a system with $C_n$-labelled glucose, must equal that of the $C_n$ position in glucose, i.e. $H_n = 1$. For the specific radioactivity of carbon 2 of hexose 6-phosphate ($H_2$) the inflow and outflow into the hexose 6-phosphate pool may be written in terms of $H_2$ and $H_5$. The outflow of hexose 6-phosphate can be shown from Fig. 5.1 to be $H_2$ (3PC + 1-PC) and the inflow $H_2PC + H_5PC$. At steady state, the outflow equals the inflow; i.e.

$$H_2(3PC + 1\text{-}PC) = H_5PC + H_2PC \qquad (5.1)$$

Rearrangement gives equation 5.2.

$$\frac{H_2}{H_5} = \frac{PC}{1 + PC} \qquad (5.2)$$

Therefore, when the pentose cycle equals the inflow, i.e. when 100% of glucose is metabolized *via* the pentose cycle, then

PC = 1 and $\frac{H_2}{H_5} = 0.5$. If the PC = 0.2 then $\frac{H_2}{H_5} = 0.167$.

In general by denoting $\frac{H_2}{H_5}$ by the term $r_{H_2,5}$ rearrangement of equation (5.1) gives:

$$PC = \frac{r_{H_2,5}}{1-r_{H_2,5}} \qquad (5.3)$$

Equation 5.3 yields a value for the pentose cycle relative to total metabolism of glucose including pathways which do not yield triose phosphates (i.e. glycogen synthesis) since no $Tr_n$ terms are required for derivation of this expression.

## 5.3 Equilibration of triose phosphate pools

The above treatment is based on the assumption that the triose phosphate end product of one turn of the cycle consists of carbons 2,3 and 2 of substrate glucose (see Fig. 5.1). It is an extension of this assumption that the dihydroxyacetone phosphate formed in reaction F (Fig. 5.1) and consisting of carbons 4,5 and 6 of glucose does not equilibrate with the product triose phosphate, but is compartmented and exclusively reacted with arabinose 5-phosphate in reaction H of Fig. 5.1 to form D-*glycero* D-*ido* octulose 1,8-bisphosphate. Equilibration of the two triose phosphate pools of the pentose cycle or their equilibration with the triose phosphates formed *via* the Embden-Meyerhof pathway would change the isotope directing predictions of the model and alter the quantitative methods for its measurement.

Evidence supporting the proposal that there is one compartment of dihydroxyacetone phosphate composed of glucose carbons 4,5 and 6 is shown by (i) the $^{14}C$-isotope distributions in hexose 6-phosphate formed from [2-$^{14}C$] glucose by isolated hepatocytes (see Chapter 7); and

(ii) the $^{14}C$-isotope distributions in glucose 6-phosphate formed *in vitro* from [1-$^{14}C$] ribose 5-phosphate by rat liver enzymes (Williams *et al.*, 1978b). If complete equilibration of the triose phosphates occurs, including those formed *via* the Embden-Meyerhof pathway, then equation 5.3 is no longer valid. In this case equating the inflow and outflow for $H_2$ yields:

$$H_2(1 + 2PC) = H_2PC + Tr_2PC \qquad (5.4)$$

$Tr_2$ can be expressed in terms of the PC and the specific radioactivities of positions 2,5 and 3 of hexose: i.e.

$$2TR_2 = H_2(1\text{-}PC) + H_5(1\text{-}PC) + H_3PC + H_5PC \qquad (5.5)$$

Inspection of Fig. 5.1 shows that C-2 or C-5 label cannot appear in C-3 of hexose, hence $H_3 = 0$ and rearrangement of eqn. 5.5 gives:

$$TR_2 = \frac{H_2(1\text{-}PC) + H_5}{2} \qquad (5.6)$$

Substituting equation 5.6 into equation 5.4 gives:

$$\frac{H_2}{H_5} = \frac{PC}{2 + PC + PC^2} \qquad (5.7)$$

Equation 5.7 shows that $\frac{H_2}{H_5}$ equals 0.25 when PC equals 1 and there is complete equilibration of the triose phosphate pools. In the absence of triose P equilibration the $\frac{H_2}{H_5}$ ratio equals 0.5. Thus in practice if triose-P equilibrium was extensive, then use of equation 5.3 would lead to a significant underestimate of the cycle. There is no case to support extensive triose phosphate pool equilibration in liver since L-type pentose cycle values calculated

using equation 5.7, yielded unacceptably high (>80%) estimates (Chapter 7).

## 5.4 Distribution of $^{14}C$ in triose phosphate following the metabolism of [2-$^{14}C$] glucose

Fig. 5.1 shows that metabolism of glucose by the L-type pentose cycle results in unique distributions of carbon atoms in triose phosphate as well as in hexose 6-phosphate. Triose phosphate formed by the aldolase cleavage of sedoheptulose 1,7-bisphosphate (reaction H, Fig. 5.1) consists of the sequence of carbon atoms 2,3 and 2 of the original glucose molecules. If [2-$^{14}C$] glucose is used as a substrate, the specific radioactivity of positions 1,2 and 3 of triose phosphates or their derivatives can be used to calculate the extent of the L-type pentose cycle relative to glycolysis. This is so because all triose phosphate formed *via* the Embden-Meyerhof pathway will contain $^{14}C$-isotope in position 2 of triose phosphate, while that formed by the L-type pentose cycle will contain isotope in carbons 1 and 3.

If it is assumed that there is equilibrium of the triose phosphates of the Embden-Meyerhof pathway and the product triose phosphate of the pentose cycle but not the triose phosphate formed by reaction F of Fig. 5.1, then the inflow and outflow in the triose phosphate pool can be expressed for $TR_1$ as:

$$TR_1(2\text{-}PC) = H_3(1\text{-}PC) + H_2PC + H_4(1\text{-}PC) \qquad (5.8)$$

Fig. 5.1 shows that no C-2 label can appear in the C-4 or C-3 positions in hexose 6-phosphate, therefore $H_3 = H_4 = 0$

and equation 5.8 becomes:

$$TR_1 = \frac{H_2PC}{2-PC} \qquad (5.9)$$

In terms of $TR_2$ the inflow and outflow can be expressed as:

$$TR_2(2-PC) = H_2(1-PC) + H_5(1-PC) + H_3PC \qquad (5.10)$$

Inspection of Fig. 5.1 shows that $H_5 = H_3 = 0$, therefore:

$$TR_2 = \frac{H_2(1-PC)}{2-PC} \qquad (5.11)$$

The ratio of the specific radioactivities of C-1 and C-2 of triose phosphate is given in equation 5.12.

$$\frac{TR_1}{TR_2} = \frac{PC}{1-PC} \qquad (5.12)$$

Denoting $\frac{TR_1}{TR_2}$ as $r_{T_{1,2}}$ then equation 5.12 is expressed as:

$$PC = \frac{r_{T_{1,2}}}{1 + r_{T_{1,2}}} \qquad (5.13)$$

Equation 5.13 may be used to determine the fraction of glucose metabolism relative to the utilization of glucose *via* the pentose cycle and Embden-Meyerhof pathway only and assumes that there is no metabolism of glucose by pathways which do not yield triose phosphates (i.e. glycogen synthesis).

It is also obvious from inspection of Fig. 5.1 that $TR_1 = TR_3$ when [2-$^{14}$C] glucose is substrate. Therefore, the distribution of $^{14}$C-isotope in the carbon atoms of triose phosphate or a derivative such as lactate or glycerol serves as a test of the validity of the reaction sequences of Fig. 5.1 since $\frac{TR_1}{TR_3}$ must be unity. It is found that the

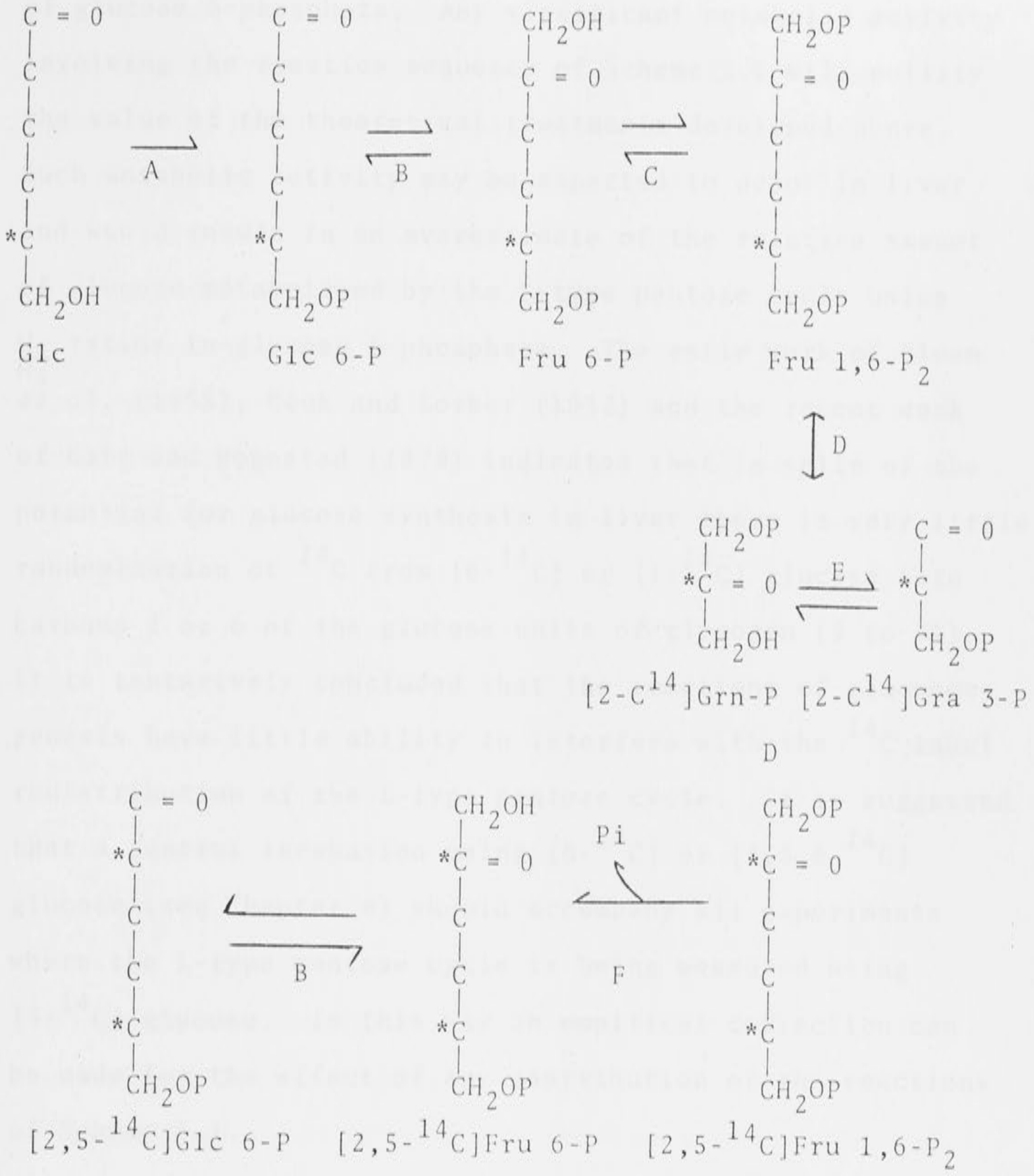
use of triose phosphate derivatives for quantitation of the L-type pentose cycle are most reliable in non-gluconeogenic tissues, i.e. erythrocytes, chloroplasts etc. Some limitations of the use of triose phosphate data are discussed in the following chapter for experiments involving measurements of the L-type pentose cycle in isolated hepatocytes.

## 5.5 Assumptions and limitations

The basic assumption on which all the preceding derivations are based requires that there are no reactions which result in carbon redistributions other than those shown in Fig. 5.1. Studies of [1-$^{14}$C] ribose 5-phosphate utilization and measurements *in vitro* of the distribution of $^{14}$C into the carbon atoms of hexose 6-phosphate (Williams *et al.*, 1978a) provided much of the evidence for the L-type pentose cycle shown in Fig. 5.1. These data also clearly indicated the extent of operation of $^{14}$C-isotope exchange reactions catalyzed by the group transferring enzymes transketolase and transaldolase. The following is an assessment of the effect of these exchange reactions and of the reactions of gluconeogenesis, non-oxidative formation of pentose 5-phosphate, isotopic equilibration of triose phosphate pools and of fructose 6-phosphate with glucose 6-phosphate, which theoretically could act to qualify a clear interpretation of data from experiments using [2-$^{14}$C] and [5-$^{14}$C] glucose.

### 5.5.1 Gluconeogenesis from triose phosphate

Theoretically the reactions of Scheme 5.1 can introduce $^{14}$C from position 5 of glucose into positions 2 and 5




Scheme 5.1:

Gluconeogenesis from triose phosphate; reactions A to E involve the phosphorylation of [5-$^{14}$C] glucose and reactions of the glycolytic sequence which lead to the aldolase catalyzed condensation of [2-$^{14}$C] glyceraldehyde 3-phosphate and [2-$^{14}$C] dihydroxyacetone phosphate to form [2,5-$^{14}$C] fructose 1,6 bisphosphate, the precursor of [2,5-$^{14}$C] glucose 6-phosphate. A = hexokinase; B = phosphoglucose isomerase; C = phosphofructokinase; D = aldolase; E = triosephosphate isomerase; F = fructose 1,6 bisphosphatase.

of glucose 6-phosphate. Any significant metabolic activity involving the reaction sequence of Scheme 5.1 will nullify the value of the theoretical treatments developed above. Such metabolic activity may be expected to occur in liver and would result in an overestimate of the relative amount of glucose metabolized by the L-type pentose cycle using $\frac{H_2}{H_5}$ ratios in glucose 6-phosphate. The early work of Bloom *et al.* (1955), Cook and Lorber (1952) and the recent work of Katz and Rognstad (1978) indicates that in spite of the potential for glucose synthesis in liver there is very little randomization of $^{14}C$ from [6-$^{14}C$] or [1-$^{14}C$] glucose into carbons 1 or 6 of the glucose units of glycogen (2 to 4%). It is tentatively concluded that the reactions of gluconeogenesis have little ability to interfere with the $^{14}C$ label redistribution of the L-type pentose cycle. It is suggested that a control incubation using [6-$^{14}C$] or [4,5,6-$^{14}C$] glucose (see Chapter 6) should accompany all experiments where the L-type pentose cycle is being measured using [5-$^{14}C$] glucose. In this way an empirical correction can be made for the effect of any contribution of the reactions of Scheme 5.1.

### 5.5.2 Reversibility of the non-oxidative segment of the pentose cycle

Previous methods for estimating the F-type pentose cycle were limited by the assumption that there was no non-oxidative formation of pentose 5-phosphate from hexose 6-P, i.e. that the transketolase and transaldolase reactions (Fig. 1.2) were not reversible. Much evidence based mainly on the metabolism [1-$^{14}C$], [2-$^{14}C$], [6-$^{14}C$] and [U-$^{14}C$]

glucose and incorporations of $^{14}C$-isotope into pentose phosphate or the pentose moiety of nucleic acid was interpreted as showing that the transketolase-transaldolase reactions were reversible (Section 4.9.2).

There are two mechanisms by which pentose 5-phosphate can be formed non-oxidatively. The first is a reversal of the coupled transketolase-transaldolase reactions shown in Fig. 1.2. The distribution of the carbon atoms of hexose 6-phosphate and triose phosphate in the resulting pentose 5-phosphate is illustrated by reaction I.

$$\begin{array}{l} H_1 \\ H_2 \\ 2\ H_3 \\ H_4 \\ H_5 \\ H_6 \end{array} + \begin{array}{l} \\ \\ T_{r_1} \\ T_{r_2} \\ T_{r_3} \end{array} \rightleftharpoons \begin{array}{l} H_1 \\ H_2 \\ T_{r_1} \\ T_{r_2} \\ T_{r_3} \end{array} + \begin{array}{l} H_1 \\ H_2 \\ H_4 \\ H_5 \\ H_6 \end{array} + \begin{array}{l} H_3 \\ H_3 \\ H_4 \\ H_5 \\ H_6 \end{array} \qquad (I)$$

2 Fru 6-P + Gra 3-P $\rightleftharpoons$ Xlu 5-P + Xlu 5-P + Rib 5-P

The alternative mechanism involves the reversal of the reactions of the L-type pentose cycle. The distribution of the carbon atoms of hexose 6-phosphate and triose phosphate are shown in reaction II.

$$\begin{array}{l} H_1 \\ H_2 \\ H_3 \\ H_4 \\ H_5 \\ H_6 \end{array} + \begin{array}{l} H_1 \\ H_2 \\ H_3 \\ H_4 \\ H_5 \\ H_6 \end{array} + \begin{array}{l} \\ \\ T_{r_1} \\ T_{r_2} \\ T_{r_3} \end{array} \rightleftharpoons \begin{array}{l} H_2 \\ H_3 \\ H_4 \\ H_5 \\ H_6 \end{array} + \begin{array}{l} T_{r_1} \\ T_{r_2} \\ H_1 \\ H_2 \\ H_1 \end{array} + \begin{array}{l} T_{r_3} \\ H_3 \\ H_4 \\ H_5 \\ H_6 \end{array} \qquad (II)$$

Fru 6-P + Glu 6-P + Grn-P $\rightleftharpoons$ Arb 5-P + Xlu 5-P + Rib 5-P

The formation of pentose 5-phosphate by either of these mechanisms, followed by the metabolism of the resulting pentose 5-phosphate *via* the mechanism of Fig. 5.1 results in $^{14}C$-isotope exchange in the absence of any net metabolism and is referred to as an exchange cycle (see Scheme 4.2). The exchange cycles will act to influence the $\frac{H_2}{H_5}$ and $\frac{Tr_1}{Tr_2}$ ratios and lead to an overestimate or an underestimate respectively of the value of PC.

It is significant that operation of reaction I results in the formation of [4-$^{14}C$] pentose 5-phosphate from [5-$^{14}C$] glucose, therefore, the redistribution of $^{14}C$-isotope in hexose 6-phosphate formed by this exchange cycle cannot be distinguished from true flux of glucose carbon through the complete L-type pentose cycle. Thus the value of PC determined using equation 5.3 represents the sum of this $^{14}C$ exchange cycle and the L-type pentose cycle. It is possible to qualitatively assess the extent of this exchange independently by using [2-$^{14}C$] glucose as substrate. The pentose 5-phosphate formed *via* the coupled transketolase-transaldolase reactions from [2-$^{14}C$] hexose 6-phosphate will contain $^{14}C$-isotope in carbon 2 (reaction I). When [2-$^{14}C$] pentose 5-phosphate is metabolized *via* the L-type pentose cycle, the $^{14}C$-isotope will be redistributed to carbon 3 of hexose 6-phosphate. Hence inspection of the $^{14}C$-isotope distribution in C-3 of hexose 6-phosphate will indicate whether or not this series of $^{14}C$ exchange reactions is active.

The operation of the $^{14}C$ exchange cycle described above will also effect the $^{14}C$-isotope distribution in derivatives of triose phosphate. The [2-$^{14}C$] pentose

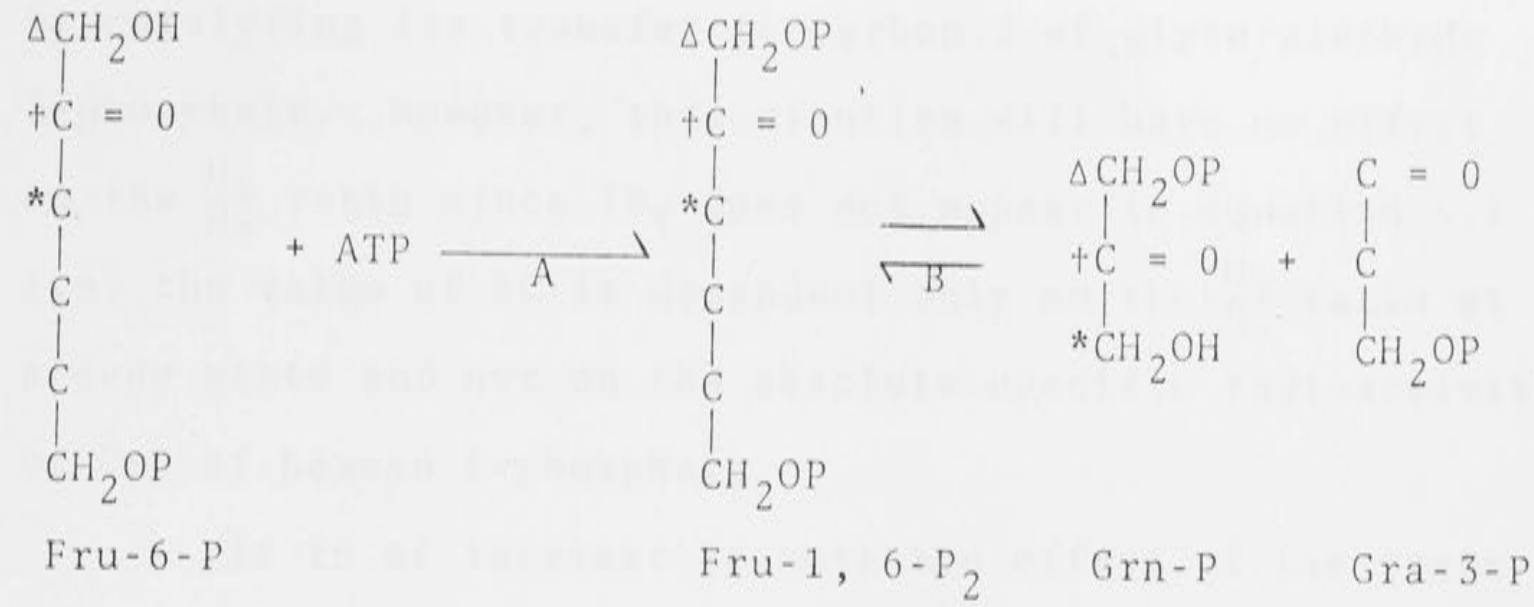
5-phosphate will result in $^{14}C$-isotope incorporation into carbon 2 of triose phosphate. Thus operation of the exchange reactions will act to reduce the value of $^{r}T_{1,2}$ and therefore the value of PC calculated from equation 5.13 will be an underestimate of the complete L-type pentose cycle.

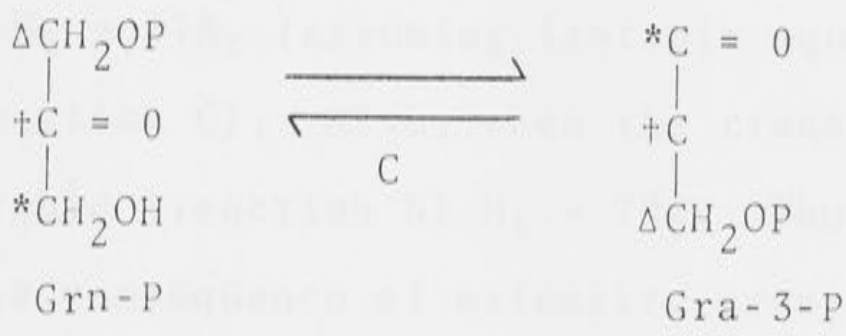
The reversibility of the non-oxidative segment of the L-type pathway results in $^{14}C$-isotope distribution which is somewhat more complex. The distribution of $^{14}C$-isotope from [5-$^{14}C$] hexose 6-phosphate cannot be distinguished from that following oxidative metabolism of hexose 6-phosphate except that the specific radioactivity of C-4 of pentose 5-phosphate is diluted by 1/3. The complexity arises from the redistribution of C-2 of hexose 6-phosphate which appears in carbons 1 and 4 of pentose 5-phosphate (reaction II). The metabolism of this pentose 5-phosphate will result in the redistribution of C-2 of the original hexose 6-phosphate into carbons 1 and 3 of triose phosphate and carbons 2 and 5 of hexose 6-phosphate. Thus $^{14}C$-isotope in $H_2$ will be lost and a fraction of it redistributed into C-5 of hexose 6-phosphate. The resulting $\frac{H_2}{H_5}$ ratio will be perturbed by the operation of these exchange reactions. Theoretically the $\frac{H_2}{H_5}$ ratio following the metabolism [5-$^{14}C$] glucose will still result in an overestimate of the L-type pentose cycle if reversible exchange of the L-type pathway occurs, since reversibility results in a greater input of $^{14}C$ from $H_5$ into $H_2$ than can be lost from $H_2$. However, the value of PC calculated from equation 5.3 must be less than the sum of pentose cycle flux plus L-type exchange.

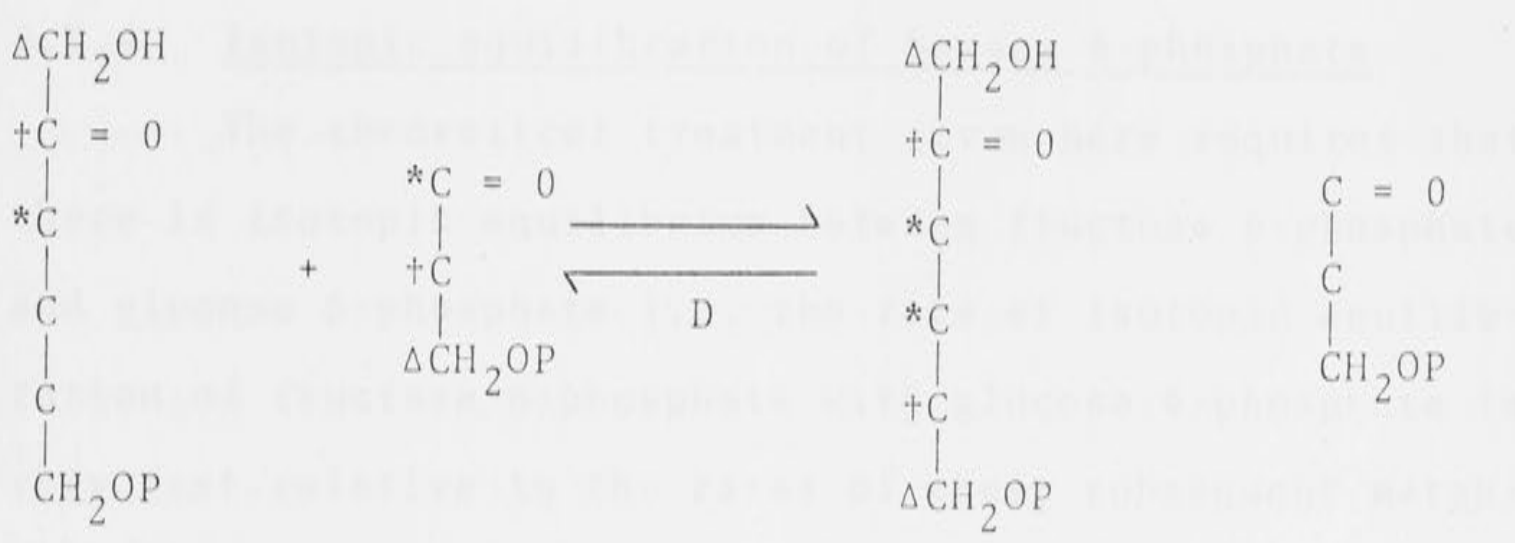
Evidence from *in vitro* experiments using a rat liver enzyme preparation and [1-$^{14}$C] and [2-$^{14}$C] glucose 6-phosphate indicates that very little pentose 5-phosphate is formed *via* reversibility of the non-oxidative segment of the L-type pathway. Following the metabolism of [1-$^{14}$C] or [2-$^{14}$C] glucose 6-phosphate and unlabelled fructose 1,6-bisphosphate (as a source of triose phosphate) by the enzymes of rat liver, the pentose 5-phosphates were isolated and the percentage distribution of $^{14}$C-isotope in each of the carbon atoms of the pentose determined. When [1-$^{14}$C] glucose 6-phosphate was substrate, 83% of $^{14}$C was found in carbon 1 of pentose 5-phosphate and 15% in carbon 5. When [2-$^{14}$C] glucose 6-phosphate was used, 85% of the $^{14}$C was located in carbon 2 of pentose 5-phosphate with 10% in carbon 3 (Williams and Blackmore, unpublished results). These results are not consistent with formation of pentose 5-phosphate *via* the reversal of the L-type pathway, (see reaction II) however, they are in reasonable agreement with the predicted carbon redistribution *via* the coupled transketolase-transaldolase reactions (reaction I).

### 5.5.3 The transaldolase exchange reaction

The activity of the reaction sequence involving the transaldolase exchange reaction (Scheme 5.II) is quite apparent from the degree of $^{14}$C-isotope labelling of carbon 5 of hexose 6-phosphate following the metabolism of [2-$^{14}$C] glucose by isolated hepatocytes (see Table 6.1 of the following chapter). The reactions of Scheme 5.II are of apparent concern since they will act to dilute the concentration of $^{14}$C-isotope in carbon 5 of hexose 6-phosphate




Scheme 5.II:

Showing the mechanism whereby carbons 1,2 and 3 of fructose 6-phosphate are incorporated into positions 6,5, and 4 of fructose 6-phosphate by reactions catalyzed by (A) phosphofructokinase, (B) aldolase, (C) triosephosphate isomerase and (D) transaldolase exchange. The reaction involving transaldolase exchange also demonstrates how $^{14}C$ from [5-$^{14}C$] fructose 6-phosphate would be diluted by incorporation into positions 2 of glyceraldehyde 3-phosphate.

by catalyzing its transfer to carbon 2 of glyceraldehyde 3-phosphate. However, this dilution will have no effect on the $\frac{H_2}{H_5}$ ratio since $TR_2$ does not appear in equation 5.1 i.e. the value of PC is dependent only on the $\frac{H_2}{H_5}$ ratio at steady state and not on the absolute specific radioactivity of C-5 of hexose 6-phosphate.

It is of interest to note the effect of the transaldolase exchange reaction when [2-$^{14}$C] glucose is substrate. From inspection of Scheme 5.II it is seen from reactions A, B and C that $H_2 = 2TR_2$ (assuming isotopic equilibrium is reached by reaction C). Also, when the transaldolase exchange is rapid (reaction D) $H_5 = TR_2$. Thus the $\frac{H_2}{H_5}$ ratio will be 2 as a consequence of extensive transaldolase exchange when [2-$^{14}$C] glucose is the substrate.

### 5.5.4 Isotopic equilibration of hexose 6-phosphate

The theoretical treatment given here requires that there is isotopic equilibrium between fructose 6-phosphate and glucose 6-phosphate i.e. the rate of isotopic equilibration of fructose 6-phosphate with glucose 6-phosphate is very fast relative to the rates of their subsequent metabolisms. This stipulation is necessary since the reactions shown in Fig. 5.1 distribute C-5 of substrate glucose into position 2 of fructose 6-phosphate. If isotopic equilibration is incomplete then the measured ratio of the specific radioactivity of position 2 relative to position 5 of glucose 6-phosphate will lead to the underestimation of the quantitative participation of the L-type pentose cycle to total glucose metabolism. Landau *et al.*, (1964) derived expressions for the estimation of the F-type pentose

cycle contribution to glucose metabolism employing a model in which no assumption was made as to the extent of isotopic equilibration between glucose 6-phosphate and fructose 6-phosphate. The application of this method to rat adipose tissue demonstrated extensive but not complete equilibration and values calculated for the percentage contribution of the pentose cycle to glucose metabolism did not differ greatly from those calculated from methods which assumed complete isotopic equilibrium (Landau and Katz, 1964). However, Till *et al.*, (1968) found slow equilibration of $^{32}P$ between glucose 6-phosphate and fructose 6-phosphate in liver and Williams *et al.*, (1971) reported a much higher specific radioactivity of fructose 6-phosphate compared with glucose 6-phosphate following the metabolism of [2-$^{14}$C] glucose by rabbit liver *in vivo*. These observations suggest that measurements of the L-type pentose cycle in liver using [5-$^{14}$C] glucose as substrate and the value of the $\frac{H_2}{H_5}$ ratio in glucose 6-phosphate together with the assumption of complete equilibration of glucose 6-phosphate and fructose 6-phosphate may underestimate the relative contribution of the cycle to total glucose metabolism.

## 5.6 Conclusions

The methods discussed above permit the investigation of the quantitative contribution of the L-type pentose cycle to the metabolism of glucose by tissues and fulfills those requirements specified in Chapter 4. The use of [2-$^{14}$C] and [5-$^{14}$C] glucose as substrates for tissues followed by the isolation and carbon atom by carbon atom

degradation of the glucose moiety of glucose 6-phosphate allows for independent estimates of the F-type and L-type pentose cycles as a consequence of the unique carbon redistributions of those two reaction sequences. Both of these estimates are independent of the rate of glucose utilization and represent the percentage of glucose metabolized *via* the pentose cycle relative to total glucose metabolism. Finally, qualitative estimates of the degree of $^{14}C$-isotope exchange reactions can be made based on the randomization of $^{14}C$-isotope in the carbon atoms of hexose 6-phosphate. Thus the expressions derived above allow for a more accurate and reasonable estimate of the L-type pentose cycle to be made than was possible using any previously available methods.

In final summary, it is of note that the metabolism of [2-$^{14}C$] and [5-$^{14}C$] glucose and the distribution of $^{14}C$ into various positions of glucose 6-phosphate and triose phosphate in tissues not only serves to measure the pathway but affords confirmatory evidence for the operation of the L-type mechanism. In using the procedures presented here for the measurement of the L-type pentose cycle, it is essential that hexose 6-phosphate and triose phosphate intermediates of the cycle should be isolated, purified and degraded for $^{14}C$-isotope analysis. The use of products of these intermediates which are metabolically distant from the pentose cycle (i.e. glucose units of glycogen instead of glucose 6-phosphate) is to be avoided since their adoption will only lead to confusion if not the inability to interpret results. This latter note of caution was clearly made by Katz and Wood (1960).

# CHAPTER 6

## MEASUREMENT OF THE L-TYPE PENTOSE CYCLE IN ISOLATED HEPATOCYTES

### 6.1 Prefatory comment

In the present experiments, the distribution of $^{14}C$-isotope in the carbon atoms of glucose 6-phosphate and lactate following the metabolism of [2-$^{14}C$], [5-$^{14}C$] and [4,5,6-$^{14}C$] glucose has been measured. The distribution of $^{14}C$-isotope in glucose 6-phosphate and lactate has been analyzed in terms of the quantitative contribution of the L-type pentose cycle to the metabolism of glucose. The extent of other carbon transferring mechanisms including gluconeogenesis from triose phosphate (using [4,5,6-$^{14}C$] glucose), transaldolase exchange reactions (using [2-$^{14}C$] glucose) (Ljungdahl *et al.*, 1961) and non-oxidative pentose 5-phosphate formation have been assessed.

### 6.2 Distribution of $^{14}C$ in glucose 6-phosphate following the metabolism of [5-$^{14}C$] glucose

The data of Table 6.1 show that 17.7% of the isotope in glucose 6-phosphate is incorporated into position 2 of that molecule following the metabolism of [5-$^{14}C$] glucose. An estimate of the percentage contribution of the L-type pentose cycle can be made from the ratio of the specific radioactivities of position 2 and position 5 of glucose 6-phosphate using the expression, $PC = r_{H_{2,5}}/1-r_{H_{2,5}}$ where $r_{H_{2,5}}$ is the ratio C-2/C-5 (Chapter 5). The C-2/C-5 ratio is 17.7-1.3 ÷ 70.8 or 0.23 and the resulting value of PC is 30%. As

Table 6.1: Distribution of $^{14}C$ in the $^{14}C$ labelled glucose substrates and in the glucose 6-phosphate isolated from liver cells after metabolism of [2-$^{14}C$], [5-$^{14}C$] and [4,5,6-$^{14}C$] glucose

Isolated hepatocytes were incubated for 1 hour at 37$^{o}$ in the presence of 5mM glucose labelled with $^{14}C$-isotope in carbons 2,5 or 4,5 and 6. Glucose 6-phosphate was isolated from $HC1O_4$ extracts of the cells and was biologically and chemically degraded to determine the specific radioactivity of each carbon atom as described in Section 2.19. The percentage recovery of label was determined by expressing the total label recovered (determined by the addition of the specific radioactivities of the six individual carbon atoms) as a fraction of the specific radioactivity obtained from the complete oxidation of the glucose moiety of glucose 6-phosphate by the method of Van Slyke and Folch (1940). A standard [1,2,6-$^{14}C$] glucose sample accompanied the degradations described in this table. Expected distribution of isotope in the standard was: C-1, 40%; C-2, 20%; C-6, 40%, distribution found; C-1, 37%; C-2, 19%; C-3, 0%; C-4, 1%; C-5, 2%; C-6, 42%. The results are the mean of the number of determinations in parentheses. The S.E.M. is shown for values based on more than 3 determinations.

Table 6.1:

| Carbon No. | Substrates | | | glucose 6-phosphate from incubations of cells with | | |
|---|---|---|---|---|---|---|
| | [2-$^{14}$C] glucose (2) | [5-$^{14}$C] glucose (2) | [4,5,6-$^{14}$C] glucose (1) | [4,5,6-$^{14}$C] glucose (2) | [2-$^{14}$C] glucose (7) | [5-$^{14}$C] glucose (4) |
| 1 | 0 | 0 | 1.2 | 2.0 | 2.5 ± 0.8 | 0.6 ± 0.2 |
| 2 | 96.5 | 1.3 | 1.6 | 12.1 | 66.8 ± 5.7 | 17.7 ± 2.7 |
| 3 | 0.4 | 0.9 | 1.7 | 2.7 | 4.2 ± 0.9 | 1.9 ± 0.2 |
| 4 | 1.2 | 2.1 | 33.0 | 30.0 | 1.8 ± 0.4 | 11.5 ± 2.7 |
| 5 | 0.6 | 94.0 | 31.4 | 24.3 | 21.8 ± 4.0 | 70.8 ± 5.6 |
| 6 | 1.0 | 1.7 | 31.0 | 28.7 | 2.9 ± 0.9 | 1.4 ± 0.3 |
| Recovery % | 87 | 93 | 87 | 96 | 84 | 87 |

previously stated (Section 5.5.2), this value represents the sum of the L-type pentose cycle contribution and "apparent" metabolism as a consequence of $^{14}C$-isotope exchange occurring due to reversibility of either a transketolase-transaldolase series of reactions or reversal of the non-oxidative segment of the L-type cycle. The latter possibility seems unlikely as previously discussed (Section 5.5.2). The role of $^{14}C$ exchange *via* the coupled transketolase-transaldolase reactions is treated in the Discussion. The data of Table 6.1 also show a significant incorporation of $^{14}C$ into position 4 of hexose 6-phosphate. This distribution of $^{14}C$ can not be explained by any known $^{14}C$-isotope transfer reactions.

## 6.3 Distribution of $^{14}C$ in glucose 6-phosphate following the metabolism of [2-$^{14}C$] and [4,5,6-$^{14}C$] glucose

Because of the potential complexity of glucose metabolism in liver, it was considered necessary to investigate the possible operation of two sets of reactions which theoretically could influence the distribution of isotope between carbon atoms 2 and 5 of hexose 6-phosphate. The two reactions are (1) the synthesis of hexose 6-phosphate from labelled triose phosphate catalyzed by aldolase and fructose bisphosphatase and (2) the transaldolase reaction which exchanges carbon atoms 4,5 and 6 of fructose 6-phosphate with glyceraldehyde 3-phosphate (Ljungdahl *et al.*, 1961). In order to estimate the contribution of these reactions which could act to qualify the measurements of the L-type pentose cycle, cells were incubated with either [2-$^{14}C$] or [4,5,6-$^{14}C$] glucose and the distribution of

$^{14}$C-isotope in the carbon atoms of glucose 6-phosphate determined as shown in Table 6.1.

The results indicate that gluconeogenesis from $^{14}$C-labelled triose phosphate in isolated hepatocytes does not significantly alter the $^{14}$C-isotope distribution in the carbon atoms of glucose 6-phosphate since very little $^{14}$C-isotope is found in positions 1 or 3. For example, the net increase in the percentage of $^{14}$C-isotope in carbon 3 of hexose 6-phosphate is only 1% which indicates that the maximum redistribution of $^{14}$C from [5-$^{14}$C] glucose into carbon 2 of hexose 6-phosphate would be approximately 3%. If this value is used to correct for the effect of gluconeogenesis, the C-2/C-5 ratio becomes $\frac{17.1-1.3-3.0}{70.8+3.0} = 0.18$ and the value of PC is 22%. It should be mentioned however that a redistribution of 1% of $^{14}$C-isotope is subject to considerable experimental error. It is of note that only position 2 of glucose 6-phosphate shows significant incorporation of $^{14}$C-isotope (10.5%) and that the greatest loss of $^{14}$C-isotope is from position 5 (7.1%). The isotope labelling pattern is consistent with the predicted redistribution of $^{14}$C *via* the L-type pentose cycle. The C-2/C-5 ratio following the metabolism of [4,5,6-$^{14}$C] glucose is higher than the ratio following the metabolism of [5-$^{14}$C] glucose. This difference may be attributed to the errors inherent in the determination of the specific radioactivities of the carbon atoms of glucose and the fact that very small changes in the percentage of isotope in position 2 results in large changes in the ratio. Since gluconeogenesis does not influence the $^{14}$C distribution in glucose 6-phosphate, it is concluded that the relocation of 21.8% of

the isotope from [2-$^{14}$C] glucose into position 5 of glucose 6-phosphate (Table 6.1) results from the transaldolase exchange reaction.

The extent of $^{14}$C incorporation into position 5 of glucose 6-phosphate following the metabolism of [2-$^{14}$C] glucose indicates that the transaldolase exchange reaction is very active under these experimental conditions. If the specific radioactivity of the triose phosphate pool is assumed to be one half that of the glucose substrate, it can be shown that isotopic equilibrium is approached between carbon atoms 4,5 and 6 of hexose 6-phosphate and the triose phosphates. Although the extent of the transaldolase exchange reaction is great and will act to dilute the specific radioactivity of position 5 of hexose 6-phosphate, this dilution will have no effect on the C-2/C-5 ratio following the metabolism of [5-$^{14}$C] glucose and therefore will not alter estimates of the L-type pentose cycle (for a discussion of the transaldolase exchange reaction see Section 5.5.3).

The distribution of $^{14}$C-isotope in the carbon atoms of glucose 6-phosphate following the metabolism of [2-$^{14}$C] glucose may also be used to calculate any contribution of the F-type pentose pathway based on the theoretical expression derived by Katz and Wood (1960). This calculation requires a measurement of the specific radioactivities of carbon atoms 1,2 and 3 of hexose 6-phosphate following [2-$^{14}$C] glucose metabolism. The data of Table 6.1 show that very little $^{14}$C-isotope is distributed into position 1 or 3 from [2-$^{14}$C] glucose and calculations using the expression of Katz and Wood (1960) result in values of 2.0

and 3.1% for the percentage of glucose metabolized *via* the F-type pentose pathway in liver. These values are in good agreement with those reported by Hostetler and Landau (1967) using rat liver *in vivo*, and indicate that there is an insignificant level of the F-type pentose cycle in liver.

## 6.4 Distribution of the $^{14}C$ in lactate following the metabolism of [2-$^{14}C$] glucose

The reaction sequence of the non-oxidative segment of the L-type pentose phosphate pathway results in a unique distribution of $^{14}C$-isotope in triose phosphate following the metabolism of [2-$^{14}C$] glucose. This redistribution results in the ratio of the specific radioactivity of carbon 1 to carbon 3 of triose phosphate being unity for any contribution of the L-type pentose cycle to glucose metabolism. An expression has been derived for estimating the percentage contribution of the L-type cycle to the metabolism of glucose relative to glycolysis based upon the ratio of the specific radioactivities of carbons 1 or 3 to carbon 2 of triose phosphate or its derivaties (Section 5.4). In practice the relatively small pool of triose phosphates makes the possibility of the isolation and degradation of triose phosphate difficult, therefore a derivative of triose phosphates such as lactate or glycerol is chosen. In liver the more accessible intermediate is lactate. In choosing lactate as the triose phosphate derivative, it is assumed that the $^{14}C$-isotope distribution in the carbon atoms of lactate reflects the distribution of $^{14}C$ in triose phosphates formed *via* the L-type pentose cycle and glycolysis only and that no other carbon transferring reactions such as $CO_2$

fixation or recycling in the tricarboxylic acid cycle occurs.

The results of Table 6.2 show the distribution of $^{14}C$-isotope in the carbon atoms of lactate following the metabolism of glucose by isolated hepatocytes. The C-1/C-3 ratio is not unity. Since the data of Table 6.2 are not consistent with the theoretical predictions on which the expression for quantitatively estimating the L-type pentose cycle were derived, the C-1/C-2 ratio in lactate cannot be used to estimate the contribution of the pentose cycle to the metabolism of glucose by isolated hepatocytes.

## 6.5 Discussion

### 6.5.1 The Mechanism of the non-oxidative segment of the pentose cycle in isolated hepatocytes

The work reported here was designed in part as prediction labelling experiments. The sequence of the reactions of the non-oxidative segment of the L-type pentose cycle proposed by Williams *et al*. (1978b) predicts the re-distribution of isotope from [5-$^{14}C$] glucose into position 2 of hexose 6-phosphate (Fig. 5.1). The reaction sequence of the F-type pathway would predict no randomization of isotope from [5-$^{14}C$] glucose. The data of Table 6.1 are entirely consistent with the operation of the L-type pentose phosphate pathway in liver, since a net increase of 16.4% of the $^{14}C$ in glucose 6-phosphate recovered from cells incubated with [5-$^{14}C$] glucose was located in position 2. The only other known mechanism for relocating $^{14}C$ from [5-$^{14}C$] glucose into position 2 of glucose 6-phosphate is gluconeogenesis from [2-$^{14}C$] triose phosphates. The extent

Table 6.2: Distribution of $^{14}C$ in the carbon atoms of lactate formed by the metabolism of [2-$^{14}C$] glucose by isolated hepatocytes

Isolated hepatocytes were incubated for 1 hour at 37$^{o}$ in the presence of 5mM [2-$^{14}C$] glucose. Lactate was isolated and purified from $HClO_4$ extracts of the cells and was chemically degraded to determine the specific radioactivity of each carbon atom as described in Section 2.19. The percentage recovery of label was determined by expressing the total label recovered (i.e. the addition of the specific radioactivities of the 3 carbon atoms) as a fraction of the specific radioactivity obtained by complete oxidation of the lactate by the method of Van Slyke and Folch (1940). The results are the mean $\pm$ S.E.M. of 7 determinations.

Table 6.2:

| Carbon No. | Percentage of Radioactivity | Ratio of Specific Radioactivities |
|---|---|---|
| 1 | 9.5 ± 0.9 | C1/C3 = 0.52 |
| 2 | 72.1 ± 2.3 | C1/C2 = 0.13 |
| 3 | 18.4 ± 1.6 | C3/C2 = 0.26 |
| Recovery % | 84 | |

of these reactions appears to be quite small since the metabolism of [4,5,6-$^{14}$C] glucose yielded a net increase of only 0.8 and 1.0% of $^{14}$C-isotope in carbons 1 and 3 of glucose 6-phosphate.

### 6.5.2 $^{14}$C-isotope distribution in lactate

The data of Table 6.2 may be interpreted in two ways. One explanation involves the possibility that the F-type and not the L-type mechanism of the cycle operates in isolated hepatocytes. The alternative explanation is that the $^{14}$C-isotope distribution in the carbon atoms of lactate does not reflect the $^{14}$C-isotope labelling pattern in triose phosphates formed *via* the pentose cycle and glycolysis in liver, rather that the $^{14}$C-isotope distribution is the consequence of the further metabolism of triose phosphate derivatives. The weight of experimental evidence favours the latter interpretation.

With respect to the mechanism of the non-oxidative segment of the pentose pathway in hepatocytes, randomization of $^{14}$C-isotope following the metabolism of [2-$^{14}$C] glucose *via* the F-type pentose cycle should introduce about twice as much $^{14}$C into position 1 of glucose 6-phosphate as position 3 and therefore the C-1/C-3 ratio in lactate should be 0.5 (Katz and Wood, 1960). Although the C-1/C-3 ratio shown in Table 2 is close to 0.5, the corresponding C-3/C-1 and C-6/C-4 ratios in glucose 6-phosphate (Table 6.1) are not consistent with the ratio found in lactate. It is a condition of the F-type pentose cycle mechanism that all $^{14}$C-isotope distribution in triose phosphate arises from glycolysis of reformed hexose 6-phosphate. Therefore, the

lack of agreement between the isotope distribution in glucose 6-phosphate and the carbon atoms of lactate indicates that lactate does not reflect the isotope distribution pattern in hexose 6-phosphate formed *via* the pentose cycle. Furthermore, the distribution of $^{14}$C-isotope in glucose 6-phosphate following [5-$^{14}$C] glucose metabolism is entirely consistent with the operation of the L-type pentose cycle. The only tissue in which the operation of the F-type pathway has been demonstrated is rat adipose tissue (Williams *et al.*, 1974). Further evidence supporting the view that there is little value in isolating and degrading metabolic intermediates which are distant from the phosphorylated intermediates of the pentose cycle is illustrated by the experiments of Katz *et al.* (1966) which showed that the $^{14}$C-isotope distribution in the carbon atoms of lactate following the metabolism of [2-$^{14}$C] glucose by rat epididymal fat pads could not be used to estimate the contribution of the pentose cycle since the C-1/C-3 ratio in the carbon atoms of lactate was twice that found in glycerol.

It may be concluded that the $^{14}$C-isotope distribution in the carbon atoms of lactate is not a reflection of the $^{14}$C-isotope distribution in triose phosphate formed as a consequence of the pentose cycle and the Embden-Meyerhof pathway. This interpretation is strengthened by the data of Grunnet and Katz (1978), Mullhofer *et al.* (1977) and Friedmann *et al.* (1971) who reported the redistribution of $^{14}$C-isotope into metabolic intermediates from [1-$^{14}$C] and [2-$^{14}$C] lactate metabolism by liver consistent with the operation of a futile cycle involving the following

reaction sequence: Lact → Pyr → OxAc → Mal → Fumarate → Mal → OxAc → PEP → Pyr → Lact. The effect of such reactions on the $^{14}$C-isotope distribution from [2-$^{14}$C] lactate would be to increase the amount of $^{14}$C-isotope in carbon 3 of lactate and thus decrease the C-1/C-3 ratio which is consistent with the data of Table 6.2.

### 6.5.3 Quantitative estimates of the pentose cycle in isolated hepatocytes

It is established (Section 5.5.2) that the value for the percentage contribution of the pentose cycle calculated from the C-2/C-5 ratio in glucose 6-phosphate is the sum of the oxidative metabolism and the contribution of $^{14}$C-isotope exchange reactions. Calculations based on $^{14}$C-isotope distribution can be misleading since they do not necessarily measure net flow of carbon, instead they frequently measure incorporation of isotope through exchange reactions. In the extreme case, the isotope distribution in glucose 6-phosphate following the metabolism of [5-$^{14}$C] glucose shown in Table 6.1 could arise entirely from the coupled transketolase, transaldolase reactions or by reversal of the reactions of the L-type pentose cycle with no glucose carbon flux through the oxidative segment of the pentose cycle.

A qualitative estimate of the extent of $^{14}$C label distribution due to isotope exchange *via* transketolase-transaldolase reactions can be made from the analysis of the distribution of $^{14}$C-isotope in the carbon atoms of glucose 6-phosphate following the metabolism of [2-$^{14}$C] glucose. Non-oxidative formation of pentose 5-phosphate

*via* the coupled transketolase, transaldolase exchange mechanism from [2-$^{14}$C] glucose yields [2-$^{14}$C] pentose 5-phosphate (Katz and Rognstad, 1967). When [2-$^{14}$C] pentose 5-phosphate is metabolized *via* the L-type pentose cycle, the $^{14}$C-isotope is redistributed into position 3 of glucose 6-phosphate. Examination of the data in Table 6.1 shows very little incorporation of $^{14}$C into position 3 of glucose 6-phosphate from [2-$^{14}$C] glucose. It is concluded that non-oxidative formation of pentose 5-phosphate *via* the coupled transketolase-transaldolase reactions does not contribute to any great extent to the redistribution of isotope in glucose 6-phosphate.

It is a theoretical condition of the method for measuring the L-type pathway that only one pool of triose phosphate contributes to the re-synthesis of hexose 6-phosphate (for a discussion of this assumption see Section 5.3). If the equilibration of the triose phosphate pools did occur, a C-2/C-5 ratio of 0.25 would represent 100% L-type pentose cycle. Furthermore, when [2-$^{14}$C] glucose was the substrate, equilibration of the two triose phosphate pools would predict a significant incorporation of $^{14}$C-isotope into position one of glucose 6-phosphate. The data of Table 6.1 support the proposition that there is little or no equilibration of the two triose phosphates formed by the reactions of the L-type pentose cycle.

The redistribution of 13 to 17% $^{14}$C-isotope from [5-$^{14}$C] glucose into position 2 of hexose 6-phosphate is largely due to the reactions of the L-type pentose cycle. The quantitative contribution of the L-type pentose cycle to the metabolism of glucose lies between 22% and 30% based

upon the ratio of the specific radioactivities of carbons 2 and 5 of glucose 6-phosphate. The contribution of $^{14}C$ exchange reactions does not appear to be large based on the redistribution of $^{14}C$ carbon 3 of hexose 6-phosphate following the metabolism of [2-$^{14}C$] glucose. Theoretically these values may represent an underestimate since it has been assumed that there is no isotopic equilibration of the pools of triose phosphate but complete isotopic equilibration of glucose 6-phosphate with fructose 6-phosphate. The values reported here are considerably higher than any previously reported for liver and show that glucose carbon flux through the L-type pentose cycle is a major component of glucose metabolism in liver. This finding is consistent with the high levels of pentose pathway enzymes found in liver (Novello and McLean, 1968) relative to the activities of enzymes which catalyze glucose catabolism by "alternate" pathways.

# CHAPTER 7

## MEASUREMENTS OF THE L-TYPE PENTOSE PHOSPHATE CYCLE IN REGENERATING LIVER AND HEPATOMA 5123TC

### 7.1 Prefatory Comment

The results of the preceding chapter indicate that the expression derived in Chapter 5 (5.3) is valid for estimating the percentage contribution of the L-type pentose cycle in liver tissue and confirmed the major assumptions involved in the derivation of the expression. The results encouraged the use of this experimental approach for examining the pentose cycle in regenerating liver and hepatoma 5123TC. The incubation conditions and experimental techniques adopted in what follows were identical to those described in the preceding chapter except that the isolation and degradation of lactate following metabolism of [2-$^{14}$C] glucose was not considered useful (see Section 6.5.2) and was not performed for these cells.

### 7.2 $^{14}$C-isotope distribution in glucose 6-phosphate following the metabolism of [5-$^{14}$C] glucose by cells from 24h regenerating liver

The data of Table 7.1 show the percentage of $^{14}$C-isotope in the carbon atoms of glucose 6-phosphate following the metabolism of [5-$^{14}$C] glucose by isolated cells. The distribution of isotope in glucose 6-phosphate isolated from cells of regenerating liver shows a similar qualitative pattern to that of normal liver with the largest percentage of redistributed $^{14}$C-isotope being located in

Table 7.1: The distribution of $^{14}C$ in the carbon atoms of glucose 6-phosphate following the metabolism of [2-$^{14}C$], [5-$^{14}C$] and [4,5,6-$^{14}C$] glucose by isolated cells from normal and 24h regenerating liver cells and hepatoma 5123TC

| Carbon No. | [2-$^{14}C$] glucose | | | [5-$^{14}C$] glucose | | | [4,5,6-$^{14}C$] glucose | |
|---|---|---|---|---|---|---|---|---|
| | N.L. (7) | R.L. (4) | 5123TC (2) | N.L. (4) | R.L. (4) | 5123TC (3) | N.L. | R.L. |
| 1 | 2.5±0.8 | 2.7±1.1 | 8.9 | 0.6±0.2 | 0.8±0.3 | 0.3 | 2.0 | 1.8 |
| 2 | 66.8±5.7 | 66.1±5.7 | 30.8 | 17.7±2.7 | 9.2±1.7 | 21.4 | 12.1 | 5.7 |
| 3 | 4.2±0.9 | 2.5±0.6 | 15.1 | 1.9±0.2 | 3.1±1.3 | 2.2 | 2.7 | 1.5 |
| 4 | 1.8±0.4 | 6.4±2.6 | 17.2 | 11.5±2.7 | 4.9±2.0 | 7.4 | 30.0 | 32.9 |
| 5 | 21.8±4.0 | 16.9±1.8 | 21.0 | 70.8±5.6 | 81.3±4.1 | 67.2 | 24.3 | 27.1 |
| 6 | 2.9±0.9 | 5.5±2.1 | 7.3 | 1.4±0.3 | 1.6±0.6 | 1.4 | 28.7 | 31.3 |
| % Recovery | 84% | 103% | 102% | 87% | 92% | 102% | 96% | 79% |

The distributions of $^{14}C$-isotope in the carbon atoms of glucose 6-phosphate following the metabolism of specifically labelled glucose by isolated cell preparations from normal and 24h regenerating liver and hepatoma 5123TC are shown. The details of the incubation conditions and degradation procedures are given in the legend to Table 6.1. The values shown are the mean±SEM for the number of determinations in parentheses.

carbon 2. Quantitatively the extent of the redistribution from carbon 5 to carbon 2 is much less in regenerating liver cells than normal liver cells. The net increase of isotope in carbon 2 is 7.9% and the resulting C-2/C-5 ratio is 0.10. Using equation 5.3, the contribution of the L-type pentose cycle is calculated to be 11%. This somewhat surprising result indicated that only 1/2 to 1/3 as much glucose was metabolized *via* the complete L-type pentose cycle by isolated hepatocytes from regenerating liver compared with cells isolated from normal adult liver in terms of total glucose metabolism.

### 7.3 $^{14}$C-isotope distribution in glucose 6-phosphate following the metabolism of [2-$^{14}$C] and [4,5,6-$^{14}$C] glucose by cells from 24h regenerating liver

The data of Table 7.1 also shows the percentage of $^{14}$C-isotope in the carbon atoms of glucose 6-phosphate following the metabolism of [2-$^{14}$C] and [4,5,6-$^{14}$C] glucose. As discussed in the preceding chapter, the use of [4,5,6-$^{14}$C] glucose allows for the estimation of the extent of the reactions of gluconeogenesis from triose-phosphate and their influence on the distribution of $^{14}$C isotope in the carbon atoms of glucose 6-phosphate. The results shown for regenerating liver indicate a net increase of isotope in carbon atoms 1,2 and 3 of glucose 6-phosphate of 0.6, 4.1 and -0.2% respectively. These results are taken to indicate that the reactions of gluconeogenesis do not influence the isotope distribution to any significant extent in regenerating liver. The observation that 4.1% of isotope is found in carbon 2 of glucose 6-phosphate is

consistent with the data when [5-$^{14}$C] glucose is substrate and indicates the operation of the L-type pentose cycle in these cells. The net increase of isotope incorporation into carbon 2 of glucose 6-phosphate from [4,5,6-$^{14}$C] glucose is 10.5% from normal liver cells which is 2.6 fold higher than that shown for regenerating cells. This result is consistent with the data for [5-$^{14}$C] glucose metabolism where the net increases in isotope incorporation are 16.4 and 7.9% for normal and regenerating cells respectively, i.e. a 2.1 fold difference.

The $^{14}$C-isotope distribution in glucose 6-phosphate following the metabolism of [2-$^{14}$C] glucose by regenerating liver cells is very similar to that of normal liver cells (Table 7.1). The net increase of isotope into carbon 5 of glucose 6-phosphate (16.3%) is indicative of an active transaldolase exchange reaction in these cells and is similar to that of normal liver (Section 6.3). There is very little isotope distributed into carbons 1 or 3 (2.7 and 1.6% respectively) which indicates very little activity of the F-type pentose cycle. Calculations of the F-type pentose cycle from C-1/C-2 and C-3/C-2 ratios (Wood *et al.*, 1963) yield values of 2.1% and 3.3% respectively. The absence of any significant $^{14}$C-isotope incorporation into C-1 and C-3 of glucose 6-phosphate also indicates that the influence of $^{14}$C exchange reactions is not a factor and that the assumption regarding separate pools of triose phosphate is probably valid based on the arguments presented in the preceding chapter.

## 7.4 $^{14}$C-isotope distribution in glucose 6-phosphate following the metabolism of [5-$^{14}$C] glucose by hepatoma 5123TC cells

The percentage of isotope in the carbons of glucose 6-phosphate isolated from hepatoma 5123TC cells following incubation with [5-$^{14}$C] glucose (Table 7.1) is very similar to that seen with normal liver. There is a net increase of 20.1% of $^{14}$C-isotope incorporated into carbon 2 of glucose 6-phosphate and very little distribution into other positions with the exception of carbon 4 (5.3%). The resulting C-2/C-5 ratio is 0.30 and the percentage contribution of the pentose phosphate cycle from equation 5.3 is 42.9%. It should be noted that this value represents the sum of L-type pentose cycle activity and $^{14}$C-isotope exchange reactions (Section 5.5.2) and is conditional on the extent of these reactions as judged by the $^{14}$C-isotope distribution following [2-$^{14}$C] glucose metabolism.

## 7.5 $^{14}$C-isotope distribution in glucose 6-phosphate following the metabolism of [2-$^{14}$C] glucose by hepatoma 5123TC cells

The data of Table 7.1 indicates a significant qualitative difference in the distribution of isotope following the metabolism of [2-$^{14}$C] glucose by the hepatoma cells relative to normal hepatocytes. The incorporation of $^{14}$C-isotope into carbon 5 is similar to that seen in normal liver and indicates the operation of an active transaldolase exchange reaction. However, a significant amount of isotope is also found in carbons 1,3,4 and 6. It is important to emphasize that the estimates of the pentose cycle

for normal and regenerating liver were largely dependent on the low levels of isotope distribution into carbon atoms other than 2 and 5. This does not appear to be the case with the hepatoma cells. The large amount of isotope incorporation into carbons 1 and 3 of glucose 6-phosphate from [2-$^{14}$C] glucose could indicate the operation of the F-type pentose cycle in this tissue and/or very rapid $^{14}$C-exchange cycle (Section 4.9.2) activity. Values of the percentage F-type pentose cycle calculated from the C-1/C-2 and C-3/C-2 ratios (Wood *et al.*, 1963) are 20.4 and 92.0% respectively. The very high percentage from the C-3/C-2 ratio indicates that the isotope redistribution is in large part due to isotope exchange reactions rather than to operation of the F-type cycle. The isotope distribution pattern is further complicated since the data from [5-$^{14}$C] glucose indicates operation of the L-type cycle in these cells which would tend to dilute the specific radioactivity of carbon 2 of glucose 6-phosphate and provide a pool of [1,3-$^{14}$C] labelled triose-phosphate which could participate in the transaldolase exchange reaction. This latter possibility may in part explain the incorporation of isotope into carbons 4 and 6 of glucose 6-phosphate.

## 7.6 $^{14}$C-isotope exchange reactions

As discussed in the preceding chapters (Sections 4.9.2, 5.5.2) the operation of specific transketolase exchange reactions and/or the exchange cycle caused by the reversibility of the non-oxidative segment of either the L or F-type pentose pathway may act to influence the isotope distribution patterns in glucose 6-phosphate and thus

complicate the interpretation of these data. In the previous chapter, the use of [2-$^{14}$C] glucose as a diagnostic substrate for the operation of these exchange reactions was treated in relation to the isotope distribution pattern in glucose 6-phosphate isolated from normal hepatocytes. In particular, it was considered that the operation of these exchange reactions would result in isotope incorporation into carbon 3 of glucose 6-phosphate from [2-$^{14}$C] glucose. The data of Tables 6.1 and 7.1 shows that these exchange reactions are not a factor in normal or regenerating liver cells. Therefore, although the percentage pentose cycle calculated from C-2/C-5 ratios using equation 5.3 represent the sum of oxidative pentose cycle flux and $^{14}$C-exchange reactions (Section 5.5.2), the values calculated for these two tissues may be considered to be reasonable estimates of the flux through the L-type pentose cycle. The situation in the tumour, however, is much more complex. The data of Table 7.1 indicates a net increase of 14.9% of $^{14}$C-isotope in carbon 3 of glucose 6-phosphate. This incorporation of isotope could arise from either the operation of the F-type pentose cycle (see Fig. 1.2) or by Reactions 7.1 and 7.2.

$$2\ [2\text{-}^{14}C]\ \text{Fru-6-P} + \text{Gra-3-P} \longrightarrow 3\ [2\text{-}^{14}C]\ \text{pentose 5-P} \quad (7.1)$$

$$3\ [2\text{-}^{14}C]\ \text{pentose 5-P} \longrightarrow 2\ [3\text{-}^{14}C]\ \text{hexose 6-P} + \text{Tri-P} \quad (7.2)$$

Reaction 7.1 is the reversal of the non-oxidative segment of the F-type pathway (Section 5.5.2) and Reaction 7.2 represents formation of hexose 6-phosphate *via* the L-type pentose pathway. If this series of reactions occurred when [5-$^{14}$C] glucose is substrate then $^{14}$C would be incorporated

into C-2 of hexose 6-phosphate and the 42.9% PC value calculated from equation 5.3 would represent the sum of the activity of the complete L-type pentose cycle plus the exchange cycle represented by Reactions 7.1 and 7.2.

A further assumption of the method involving [5-$^{14}$C] glucose metabolism by tissues is that only one pool of triose phosphate contributes to the resynthesis of hexose 6-phosphate from pentose 5-phosphate (Section 5.3). [2-$^{14}$C] glucose has been used as a diagnostic substrate for testing this assumption since equilibration of the two pools of triose-phosphate formed by the L-type pentose cycle (Fig. 5.1) would result in $^{14}$C-isotope incorporation into carbon 1 of glucose 6-phosphate. The data of Tables 6.1 and 7.1 indicate that little or no equilibration of triose-phosphate pools occurs in normal or regenerating liver cells since the amount of isotope incorporated into carbon 1 of glucose 6-phosphate is small in these tissues (i.e. 2.5 and 2.7% respectively). However, there is a significant incorporation of isotope into carbon 1 of glucose 6-phosphate isolated from the hepatoma cells (8.9%) following the metabolism of [2-$^{14}$C] glucose. This incorporation may be the result of triose-phosphate pool equilibration, however it should be recognized that it may also result from operation of the F-type pentose cycle. As one is unable to distinguish between these two options, further interpretation of the labelling pattern is rendered difficult if not impossible.

The distribution of isotope from [2-$^{14}$C] glucose into carbon 5 of glucose 6-phosphate is an indicator of the extent of the transaldolase exchange reaction (Section 5.5.3).

The data of Table 7.1 indicates that this reaction is very active in all three cell types with 21.8, 16.9 and 21.0% of $^{14}C$-isotope found in carbon 5 of normal, regenerating and hepatoma cells respectively. The significant incorporation of isotope in carbon atoms 4 and 6 of glucose 6-phosphate from 5123TC hepatoma cells reflects the $^{14}C$-isotope in carbons 3 and 1 respectively and further supports the proposal of a rapid activity of this exchange reaction and of the equilibration of triose-phosphates in this tissue.

## 7.7 Anomalous $^{14}C$-isotope distributions

A careful scrutiny of the $^{14}C$ distribution in the carbon atoms of glucose 6-phosphate reveals two features which cannot be explained based on the preceding discussion. The first is the incorporation of a large percentage of isotope in C-4 of glucose 6-phosphate after [5-$^{14}C$] metabolism by hepatocytes. No satisfactory explanation for the redistribution of isotope into this position is apparent. The second observation is the failure to find any net accumulation of isotope in C-1 of glucose 6-phosphate following [4,5,6-$^{14}C$] glucose metabolism. The use of [4,5,6-$^{14}C$] glucose in these experiments was intended primarily as an estimate of the reactions of gluconeogenesis and the low levels of isotope found in C-1 and C-3 were taken to indicate that these reactions did not contribute significantly to isotope redistribution. However, it is evident from Fig. 5.1 that $^{14}C$-isotope should be incorporated into C-1 from both C-4 and C-6 of glucose when [4,5,6-$^{14}C$] glucose is substrate. The predicted net

accumulation of isotope in C-1 is compromised by recycling the reformed hexose 6-phosphate through the pentose cycle since all $^{14}C$ in C-1 will be lost as $^{14}CO_2$, nevertheless closer analysis (see the Appendix) indicates that a significant amount of isotope should accumulate in C-1. There is no explanation for the absence of any net accumulation as shown in Table 7.1. These observations indicate that the metabolism of glucose by liver cannot be entirely explained by the reaction sequences discussed in this thesis.

## 7.8 Conclusions

The use of [2-$^{14}C$] and [5-$^{14}C$] glucose as diagnostic substrates for the F-type and L-type pentose cycle mechanisms has been discussed in a preceding chapter (Section 5.6). The data of Table 7.1 indicates that the L-type pentose cycle is the major variant of the cycle in both normal and regenerating liver. This conclusion is based on the extent of $^{14}C$ in carbon 2 of hexose 6-phosphate following the metabolism of [5-$^{14}C$] glucose and the very low amounts of isotope in carbons 1 and 3 after [2-$^{14}C$] glucose metabolism. The estimate of the pentose cycle in regenerating cells is 11% and is unqualified with respect to isotope exchange reactions. The situation in the hepatoma cells, however, is more complex. The incorporation of isotope into carbon 2 of glucose 6-phosphate following [5-$^{14}C$] glucose metabolism indicates a significant operation of the L-type pentose cycle in these cells. Assuming no gluconeogenic activity, the incorporation of 20.1% of $^{14}C$-isotope into carbon 2 could only arise as a consequence of the formation of glucose 6-phosphate from [4-$^{14}C$]

pentose 5-phosphate *via* the non-oxidative segment of the L-type pentose cycle. However, the [4-$^{14}$C] pentose 5-phosphate could arise from a number of reaction sequences including non-oxidative formation *via* transketolase and transaldolase and $^{14}$C-exchange reactions. The $^{14}$C distributions in carbons 1 and 3 of glucose 6-phosphate following [2-$^{14}$C] glucose metabolism may be taken to be a consequence of the operation of the F-type pentose cycle in this tissue, although the C-1/C-3 ratio of 0.6 indicates that other reactions largely contribute to the accumulation of isotope in carbon 3. The percentage L-type pentose cycle is 42.9% for the hepatoma, however as this value is not unqualified with respect to exchange reactions, it may be considered to be an over-estimate of the actual flux of carbon through the L-type pentose cycle and reflects instead an extensive contribution of exchange reactions. Thus the quantitative contribution of the pentose cycle in hepatoma cells remains unclear and there is little certainty that the problems will be easily resolved.

# CHAPTER 8

# GENERAL DISCUSSION

## 8.1 Introduction

The experimental work described in this thesis has been based on the assumption that the major physiological role of the pentose phosphate pathway is the production of metabolic intermediates useful or necessary for biosynthesis. It was considered that growth conditions in hepatic tissues would be accompanied by increased metabolic flux through the pathway as a necessary prerequisite to cell growth and division. The analysis of this proposition has required experiments designed to elucidate the mechanism or sequence of reactions of the pathway in the various tissues under study prior to attempts to quantitatively assess the contribution of the pathway using $^{14}C$-isotope methods. The data presented in the preceding chapters is open to a number of possible interpretations. It is the intent of this final discussion to collate the results presented in the foregoing chapters in an attempt to gain a clearer understanding of the significance of glucose metabolism through the pentose phosphate pathway in growing hepatic tissues.

## 8.2 Reversibility of the non-oxidative segment of the pentose phosphate pathway and $^{14}C$-isotope exchange reactions

The interpretation of all isotope experiments discussed in the preceding chapters has been based on the

assumption that the direction of net glucose carbon flux is from hexose to pentose in the oxidative segment of the pentose pathway and from pentose to hexose in the non-oxidative segment. This assumption is based largely on the results of the experiments *in vitro* of Williams and Blackmore (unpublished results) which demonstrated very little net formation of pentose phosphate from the incubation of [1-$^{14}$C] and [2-$^{14}$C] glucose 6-phosphate and fructose 1,6 bisphosphate with an enzyme preparation of rat liver (see Section 4.9.2). Further evidence supporting this view is provided by the analysis of $^{14}$C-isotope distribution in the carbon atoms of pentose 5-phosphate and hexose following the metabolism of [1-$^{14}$C] and [2-$^{14}$C] glucose by a variety of cell types as discussed by Katz and Rognstadt (1967). The reversibility of the non-oxidative segment of the cycle and/or $^{14}$C-isotope exchange reactions, however, remain areas of some controversy in the literature as previously discussed (Section 4.9.2). Since the potential for operation of these reactions may compromise estimates of the L-type pentose cycle it was considered pertinent at this point to discuss the possibilities and their influence in detail prior to attempting any analysis of the pentose cycle in tissues.

A number of workers have concluded that in many organisms pentose phosphate is formed by both the oxidative and non-oxidative (F-type) pentose pathways and that the pentose cycle does not operate. This conclusion is based largely on the observed incorporation of $^{14}$C-isotope into the pentose moiety of nucleic acids from [1-$^{14}$C] glucose. The extent of $^{14}$C-isotope incorporation into nucleic acids

from [1-$^{14}$C] and [6-$^{14}$C] glucose discussed in Chapter 4 is in good agreement with the results of other workers, however these results were interpreted as indicating the operation of a series of $^{14}$C-exchange reactions (Scheme 4.2) rather than the net non-oxidative synthesis of pentose phosphate. It was considered in Chapter 5, that the operation of $^{14}$C-exchange reactions could act to influence the quantitation of the L-type pentose cycle and the use of [2-$^{14}$C] glucose as a diagnostic substrate was proposed. The [2-$^{14}$C] glucose was chosen since non-oxidative formation of pentose 5-phosphate *via* reversal of the F-type pathway results in [2-$^{14}$C] pentose 5-phosphate. The metabolism of [2-$^{14}$C] pentose 5-phosphate *via* the L-type pathway results in [3-$^{14}$C] hexose 6-phosphate formation. The assumption that hexose 6-phosphate is formed from pentose 5-phosphate *via* the L-type mechanism was considered valid in light of the $^{14}$C-isotope distribution in glucose 6-phosphate following the metabolism of [5-$^{14}$C] glucose. The results of Table 7.1 do not show any significant incorporation of $^{14}$C-isotope into carbon 3 of glucose 6-phosphate from [2-$^{14}$C] glucose, thus it was concluded that $^{14}$C-isotope exchange *via* reversal of the transketolase-transaldolase-transketolase reaction sequence is not a significant factor in determining the distribution of $^{14}$C in reformed hexose 6-phosphate.

It therefore appeared that the results of Chapter 4 and Chapter 7 were in conflict. On the one hand it is necessary to propose the extensive operation of $^{14}$C-isotope exchange reactions to explain the incorporation of $^{14}$C into nucleic acids from [1-$^{14}$C] glucose and on the other the $^{14}$C-isotope distribution in the carbon atoms of glucose

6-phosphate following the metabolism of [2-$^{14}$C] glucose indicated that very little $^{14}$C-isotope exchange occurred, at least *via* the transketolase-transaldolase-transketolase reaction sequence. This inconsistency in the results necessitates a re-examination of the possibilities for $^{14}$C-isotope exchange in search of a reaction sequence which would explain how $^{14}$C could be incorporated into nucleic acids from [1-$^{14}$C] glucose and still be consistent with the isotope distribution pattern in reformed hexose 6-phosphate. Further, it was necessary to assume that the hexose 6-phosphate was reformed *via* the L-type reaction sequence. It is clear that any reaction sequence or exchange reactions which result in formation of [2-$^{14}$C] pentose 5-phosphate from [2-$^{14}$C] glucose will not satisfy these requirements. Therefore, the reversal of the F-type pentose pathway and the transketolase reaction between fructose 6-phosphate and xylulose 5-phosphate (Reaction 4.1) are not possibilities. The operation of the transketolase reaction between sedoheptulose 7-phosphate and xylulose 5-phosphate (Reaction 4.3) will not result in $^{14}$C incorporation into pentose from [1-$^{14}$C] glucose and therefore is also eliminated as a possibility.

The transketolase exchange reaction between xylulose 5-phosphate and glyceraldehyde 3-phosphate (Reaction 4.2) however, does not result in [2-$^{14}$C] pentose 5-phosphate from [2-$^{14}$C] glucose and warrants further consideration. In the following discussion the carbon atoms of hexose are represented as H1, H2 etc., of pentose as P1, P2 etc., and of triose-P as TR1, TR2 and TR3. The distribution of carbon atoms resulting from the transketolase exchange

reaction are shown in equation 8.1.

$$\begin{matrix} \text{P1} \\ \text{P2} \\ \text{P3} \\ \text{P4} \\ \text{P5} \\ \text{Xlu 5-P} \end{matrix} \quad + \quad \begin{matrix} \text{TR1} \\ \text{TR2} \\ \text{TR3} \\ \text{Gra-P} \end{matrix} \quad \rightleftharpoons \quad \begin{matrix} \text{P1} \\ \text{P2} \\ \text{TR1} \\ \text{TR2} \\ \text{TR3} \\ \text{Xlu 5-P} \end{matrix} \quad + \quad \begin{matrix} \text{P3} \\ \text{P4} \\ \text{P5} \\ \text{Gra-P} \end{matrix} \qquad (8.1)$$

If it is assumed that the origin of the carbon atoms of the triose phosphate is largely a consequence of the metabolism of glucose *via* the glycolytic pathway then the following substitution can be made (8.2):

$$\begin{matrix} \text{TR1} \\ \text{TR2} \\ \text{TR3} \end{matrix} \quad = \quad \begin{matrix} \text{H3,4} \\ \text{H2,5} \\ \text{H1,6} \end{matrix} \qquad (8.2)$$

The resulting pentose 5-phosphate formed will then consist of the carbon atoms as shown in 8.3.

$$\begin{matrix} \text{P1} \\ \text{P2} \\ \text{H3,4} \\ \text{H2,5} \\ \text{H1,6} \end{matrix} \qquad (8.3)$$

It is also convenient to express the carbon atoms P1 and P2 in terms of their hexose precursors. Since non-oxidative formation of pentose 5-phosphate will result in [2-$^{14}$C] pentose 5-phosphate from [2-$^{14}$C] glucose, only pentose 5-phosphate formed *via* the oxidative segment of the pathway or endogenous pentose are candidates for the exchange reaction, thus P1 = H2 and P2 = H3 and the pentose 5-phosphate can be re-expressed in terms of hexose carbons as shown in 8.4.

H2
H3
H3,4
H2,5
H1,6 (8.4)

The metabolism of this pentose 5-phosphate *via* the L-type pentose pathway will result in hexose 6-phosphate as shown in 8.5.

| | | |
|---|---|---|
| H6,1 | | H4,3 |
| H5,2 | | H2 |
| H3 | and | H3 |
| H4,3 | | H4,3 |
| H5,2 | | H5,2 |
| H6,1 | | H6,1 (8.5) |

In this series of reactions, carbon atoms 2 and 5 of hexose 6-P both contribute to position 2 of triose-P, thus [2-$^{14}$C] and [5-$^{14}$C] glucose will both yield [2,5-$^{14}$C] glucose 6-phosphate which is consistent with the data of Table 7.1. It is also clear from 8.4 that $^{14}$C-isotope may be incorporated into nucleic acid pentose to the same extent from both [1-$^{14}$C] and [6-$^{14}$C] glucose if the operation of this exchange reaction is very active relative to pentose cycle flux.

Although the operation of this exchange reaction will obviously affect the C-2/C-5 ratio in hexose 6-phosphate following the metabolism of [5-$^{14}$C] glucose, it will not change the estimate of the percentage contribution of the L-type pentose cycle since it is a prerequisite that the pentose 5-P participating in the exchange reaction is formed *via* the oxidative segment of the cycle. Further any dilution of the $^{14}$C-isotope in carbon 4 of pentose resulting from the exchange reaction will be reflected in both carbon 5 and carbon 2 of reformed hexose 6-phosphate and

therefore will not change the ratio.

Thus the operation of the transketolase exchange reaction (8.1) followed by metabolism of the pentose 5-phosphate *via* the L-type pentose cycle is consistent with the $^{14}C$-isotope data reported in this thesis. The predicted $^{14}C$-isotope distribution in the carbon atoms of pentose 5-phosphate (8.4) however, is not consistent with those reported by other workers. Hiatt (1957) measured the distribution of $^{14}C$-isotope in the pentose moiety of RNA of HeLa cells grown in media containing either [1-$^{14}C$] or [2-$^{14}C$] glucose as shown below:

| Carbon No. | [1-$^{14}C$] glucose | [2-$^{14}C$] glucose |
|---|---|---|
| 1 | 52.8% | 23.3% |
| 2 | 4.9 | 37.0 |
| 3 | 8.2 | 11.6 |
| 4 | 0.5 | 22.3 |
| 5 | 33.6 | 5.8 |

Although these results are not from hepatic tissue (and therefore an alternative pathway may be acting in these cells), they are not what might be expected from the reaction sequence discussed above. The metabolism of [1-$^{14}C$] glucose should result in $^{14}C$ only in C-5 of pentose (see 8.4) whereas the data shows more than 1/2 the total isotope in C-1. The metabolism of [2-$^{14}C$] glucose should yield [1,4-$^{14}C$] pentose, and although 45.6% of isotope is located in these two carbon atoms, an additional 37% is located in carbon 2. The incorporation of $^{14}C$ into C-1 of pentose from [1-$^{14}C$] glucose and into C-2 from [2-$^{14}C$] glucose is consistent with the operation of the transketolase-transaldolase-transketolase sequence of reactions.

Horecker *et al.* (1958) administered [2-$^{14}C$] glucose

to rats every 6h for 56h following 90% hepatectomy and isolated and degraded RNA-ribose, DNA-deoxyribose and glycogen from the regenerating liver. The $^{14}C$ in these sugars were found to be distributed as shown below:

| Carbon No. | RNA ribose | DNA deoxyribose | Glycogen-glucose |
|---|---|---|---|
| 1 | 28% | 32% | 15% |
| 2 | 42 | 31 | 42 |
| 3 | 9 | 11 | 9 |
| 4 | 15 | 14 | 14 |
| 5 | 7 | 11 | 12 |
| 6 | - | - | 9 |

These results show that C2 of pentose 5-P contains a significant amount of $^{14}C$, however very little $^{14}C$ was found in C-3 of glucose in glycogen. The $^{14}C$ in C-1 and C-4 of pentose is consistent with the operation of the transketolase exchange reaction discussed above. It is of note that the $^{14}C$-labelling pattern in the hexose shown above is not consistent with the data of Table 7.1. The use of this *in vivo* system and the length of time the animal was exposed to the $^{14}C$-labelled glucose makes analysis of this data very complex since it is certainly true that some fraction of the $^{14}C$ glucose was metabolized by tissues other than the liver and the $^{14}C$ may then have been presented to the liver as $^{14}C$-lactate or $^{14}C$-lipid. Thus a complex randomization of $^{14}C$ could have occurred prior to its incorporation into glycogen or nucleic acid.

The question of how $^{14}C$-isotope is incorporated into pentose 5-phosphate is central to the argument of whether or not there is any net flux in the direction of pentose 5-phosphate formation *via* non-oxidative mechanisms. As previously discussed, the net formation of pentose

5-phosphate *in vitro* is very small and the distribution of isotope in the small amounts of pentose 5-phosphate formed indicated that it was formed *via* the reversal of the F-type mechanism (see Section 4.9.2). The results discussed above suggest that for cell preparations this series of reactions does not contribute significantly to the formation of pentose 5-phosphate in liver, although it may be responsible for formation of pentose in HeLa cells. The transketolase catalyzed reaction between xylulose 5-phosphate and glyceraldehyde 3-phosphate, however, will account for the results presented here and may also play a role in the distribution of isotope in HeLa cells. There is an important distinction between the reversal of the F-type pathway, which may lead to net pentose 5-phosphate formation, and the transketolase exchange reaction in which there can be no net formation of pentose 5-phosphate since the reactants and products are the same and only isotope is exchanged. Since the transketolase exchange reaction (8.1) is more consistent with the data described in this thesis, it is tentatively concluded that this reaction is largely responsible for the incorporation of $^{14}C$-isotope into pentose 5-phosphate and nucleic acid and therefore there is no net synthesis of pentose 5-phosphate by non-oxidative reactions in liver.

## 8.3 Anomalous observations

The results presented here have been interpreted largely within the framework of the reaction schemes shown in Figs. 1.2 and 1.3. The curious appearance of $^{14}C$-isotope in C-4 of glucose 6-phosphate following [5-$^{14}C$] glucose

metabolism by isolated hepatocytes (Chapter 6), and the failure to find any $^{14}C$ in C-1 of glucose 6-phosphate after the metabolism of [4,5,6-$^{14}C$] glucose are inexplicable in terms of these reaction schemes or any other known reactions of the intermediates of the pentose pathway. These results suggest that other as yet unknown reactions exist for the metabolism of these intermediates. The formation of hexose 6-phosphate from *altro*-heptulose-7-phosphate as sole substrate (Chapter 3) is a case in point. This observation has been previously reported (Williams *et al.*, 1978b) and was considered to be the result of a coupled transketolase-transaldolase reaction giving rise to fructose 6-phosphate and octulose 8-phosphate. The potential for this reaction has recently been confirmed using commercial preparations of transketolase and transaldolase (from yeast) (Cortis, Williams and Williamson, unpublished results) although its quantitative significance in liver is unknown. The formation of hexose 6-phosphate by liver enzymes from erythrose 4-phosphate has also been observed. The reaction mechanism involved is untested, however it could be explained by the interconversion of D-erythrose 4-phosphate with L-erythrulose 4-phosphate followed by a transketolase catalyzed reaction between these two tetrose phosphates to yield fructose 6-phosphate and glycoaldehyde phosphate. Neither of these two reactions can explain the anomalous $^{14}C$-isotope distributions mentioned above.

The metabolism of arabinose 5-phosphate by liver enzymes appears to be complex. In addition to the inhibition of transaldolase previously reported (Williams *et al.*, 1978b), the rate of triose phosphate formation from arabinose

5-phosphate has been shown to be very concentration dependent (Fig. 8.1) and the rate of hexose 6-phosphate formation from ribose 5-phosphate is inhibited in the presence of arabinose 5-P (Fig. 8.2) (Williams and co-workers, unpublished results). Further, it has recently been established that a product of arabinose 5-phosphate metabolism by rat liver enzymes is a potent inhibitor of glucose 6-phosphate dehydrogenase. The metabolism of arabinose is further complicated by the observation that D-arabinose is converted to L-lactate and glycollate by enzymes of porcine liver (Chan, J.Y., Nwokoro, N.A. and Schachter, H. (1979) J. Biol. Chem. 254, 7060-7068). Thus the metabolism of arabinose 5-phosphate and its role in the regulation of flux through the oxidative segment of the pentose pathway adds complexity to the already intricate mechanisms of regulation discussed in Section 1.2.1.

The metabolism of arabinose 5-phosphate, the octulose phosphates and *manno*-heptulose 7-phosphate are all areas where very little is known. It is considered that elucidation of the metabolism of these sugar phosphates, particularly in relation to the arabinose 5-phosphate epimerase and the phosphotransferase enzymes is a necessary step to understanding the significance and regulation of the pentose phosphate pathway and cycle.

Since the interpretation of $^{14}C$-isotope data discussed in the preceding chapters is dependent on the predicted randomization of the carbon atoms of glucose 6-phosphate as it is metabolized *via* the pentose pathway, a complete understanding of the metabolism of the intermediates of the pathway is essential to a "true"

Fig. 8.1: The rate of formation of glyceraldehyde 3-phosphate from arabinose 5-phosphate by rat liver enzymes

The figure shows the rates of glyceraldehyde 3-phosphate formation as a function of arabinose 5-phosphate concentration by an acetone-dried powder extract of rat liver enzymes. The rate of glyceraldehyde 3-phosphate formation was determined spectrophotometrically by recording the change in optical density at 340nm. The data shown are unpublished results of Williams and co-workers (manuscript in preparation).

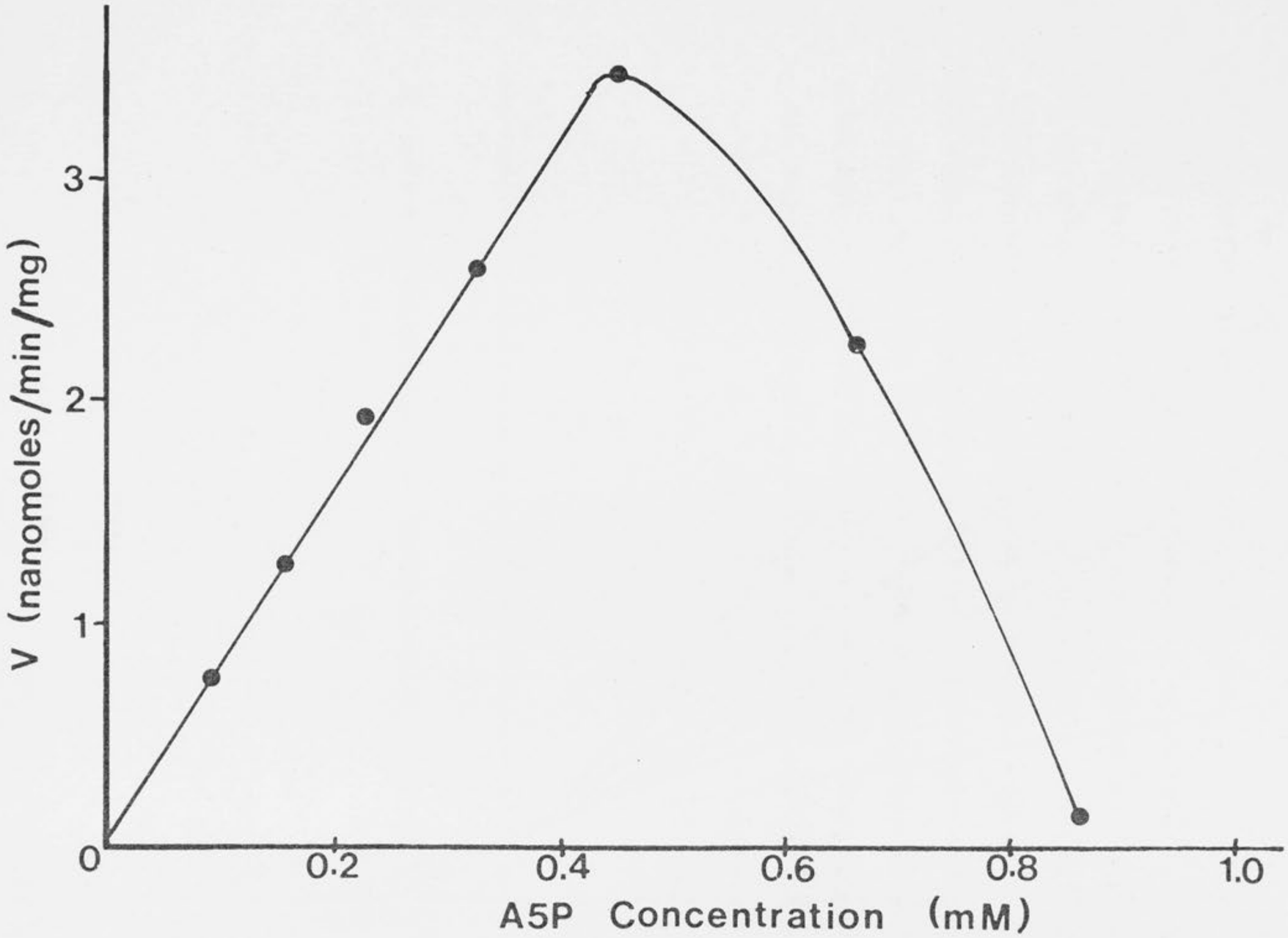



Fig 8.1

Fig. 8.2: The effect of arabinose 5-phosphate on the rate of hexose 6-phosphate formation from ribose 5-phosphate by rat liver enzymes

The rate of hexose 6-phosphate formation from ribose 5-phosphate was determined using the procedure described in Section 2.11 except that the enzyme preparation used was a buffered extract of an acetone-dried powder of rat liver. Arabinose 5-phosphate inhibition of the rates were determined by the addition of this sugar phosphate to the incubation at concentrations of 0, (x); 0.1mM, (●); 0.25mM, (□); 0.5mM (⊕); and 1mM, (○). The data shown are from Williams and co-workers (manuscript in preparation).

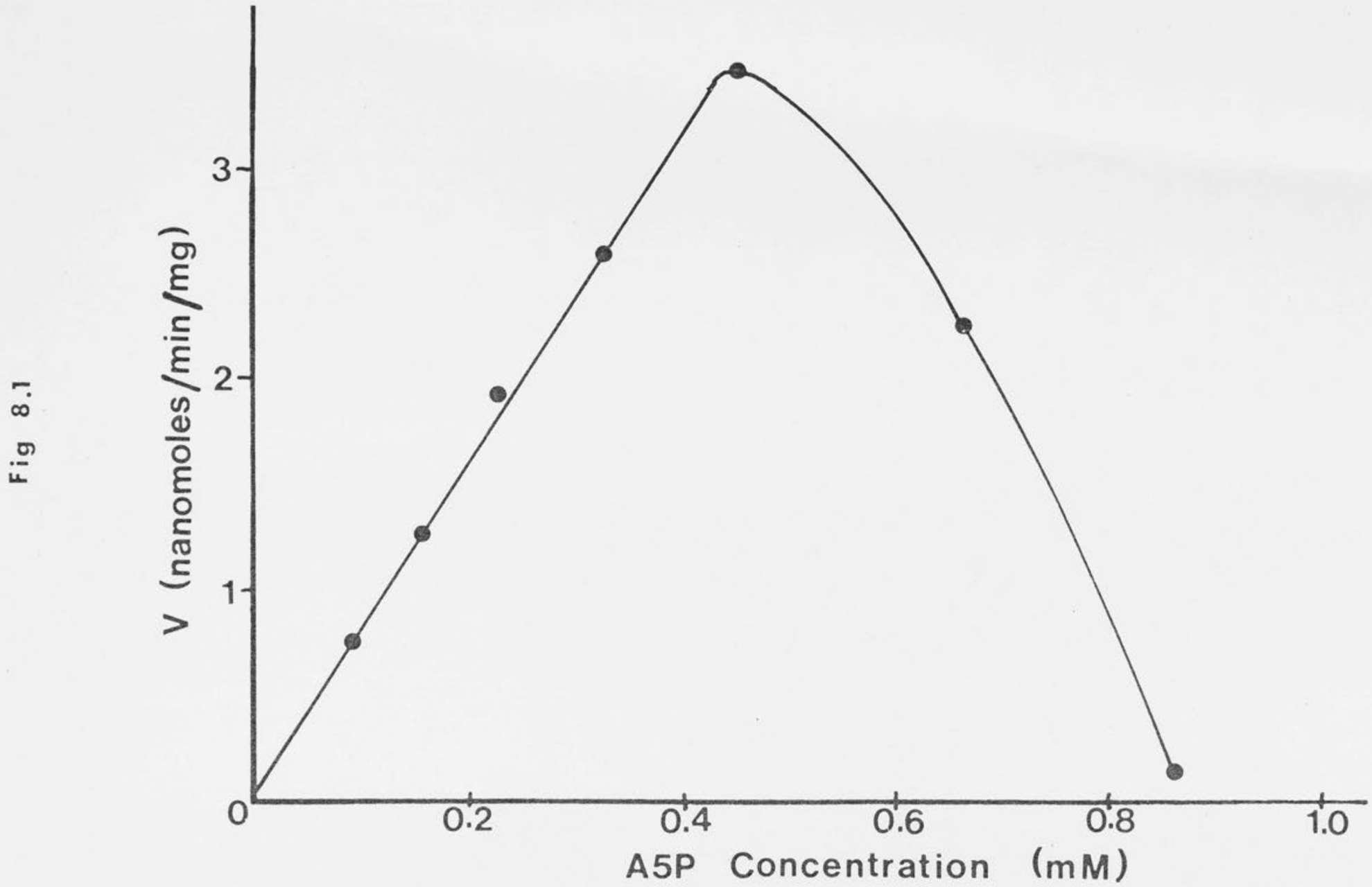
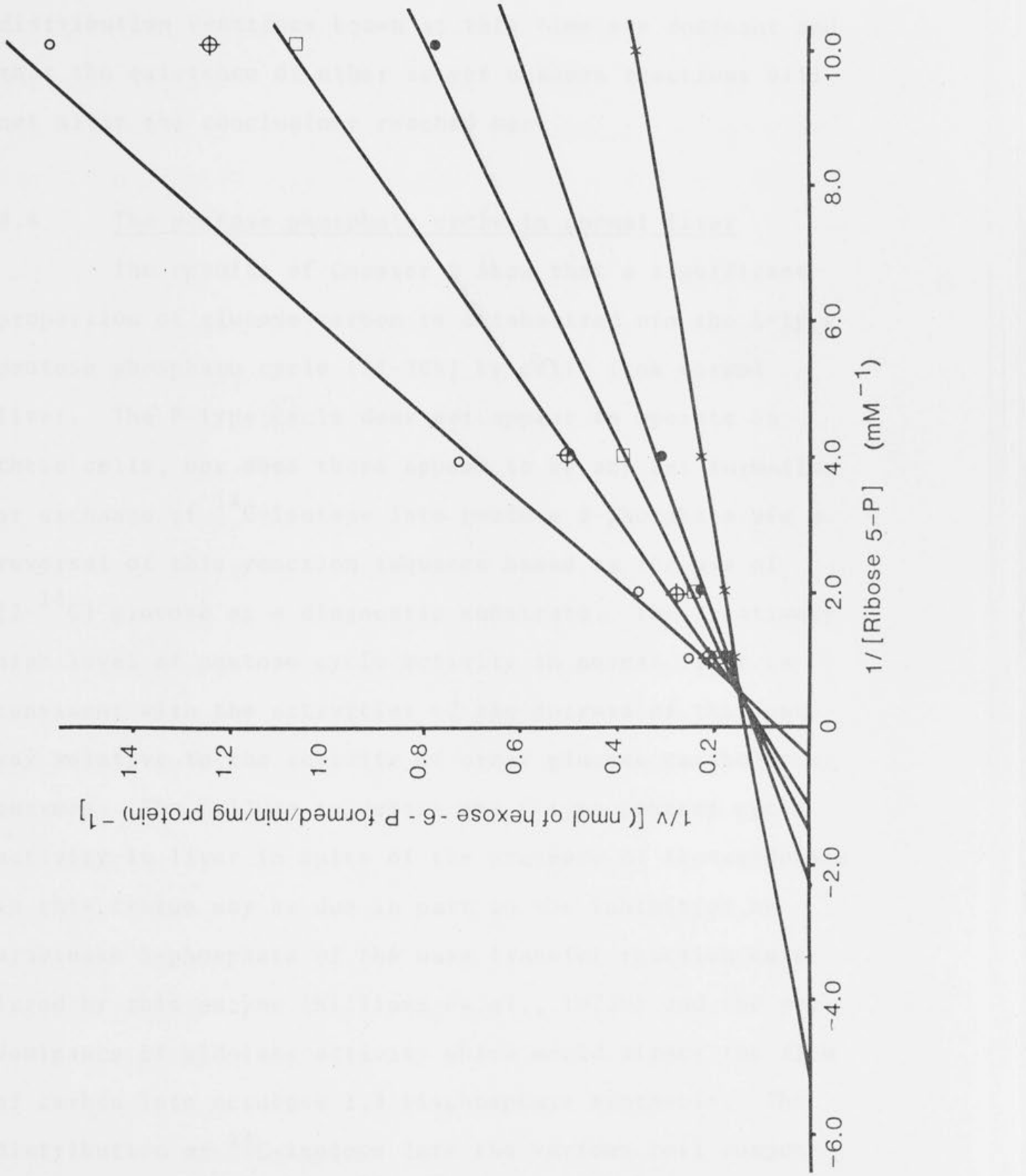



Fig 8.1

Fig. 8.2: The effect of arabinose 5-phosphate on the rate of hexose 6-phosphate formation from ribose 5-phosphate by rat liver enzymes

The rate of hexose 6-phosphate formation from ribose 5-phosphate was determined using the procedure described in Section 2.11 except that the enzyme preparation used was a buffered extract of an acetone-dried powder of rat liver. Arabinose 5-phosphate inhibition of the rates were determined by the addition of this sugar phosphate to the incubation at concentrations of 0, (x); 0.1mM, (●); 0.25mM, (□); 0.5mM (⊕); and 1mM, (○). The data shown are from Williams and co-workers (manuscript in preparation).

Fig 8.2


1/v [( nmol of hexose -6 - P formed/min/mg protein) $^{-1}$]
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
-2.0
-4.0
-6.0
2.0
4.0
6.0
8.0
10.0
1/ [Ribose 5-P] (mM$^{-1}$)

interpretation of the data. Thus the interpretations discussed here must be acknowledged to be tentative and dependent upon the validity of the assumption that the $^{14}C$-distribution reactions known at this time are dominant and that the existence of other as yet unknown reactions will not alter the conclusions reached here.

## 8.4 The pentose phosphate cycle in normal liver

The results of Chapter 6 show that a significant proportion of glucose carbon is catabolized *via* the L-type pentose phosphate cycle (22-30%) by cells from normal liver. The F-type cycle does not appear to operate in these cells, nor does there appear to be any net formation or exchange of $^{14}C$-isotope into pentose 5-phosphate *via* a reversal of this reaction sequence based on the use of [2-$^{14}C$] glucose as a diagnostic substrate. The relatively high level of pentose cycle activity in normal liver is consisent with the activities of the enzymes of this pathway relative to the activity of other glucose catabolizing enzymes. The failure to detect any F-type pentose cycle activity in liver in spite of the presence of transaldolase in this tissue may be due in part to the inhibition by arabinose 5-phosphate of the mass transfer reaction catalyzed by this enzyme (Williams *et al.*, 1978b) and the predominance of aldolase activity which would direct the flow of carbon into octulose 1,8-bisphosphate synthesis. The distribution of $^{14}C$-isotope into the various cell components from [1-$^{14}C$] and [6-$^{14}C$] glucose (Chapter 4) are in reasonable agreement with the expected distribution (Katz *et al.*, 1975) and provide a basis for comparison with the

regenerating liver and hepatoma 5123TC results. The results of Chapter 4 indicate very little glucose carbon is directed into nucleic acid in normal liver cells which supports the argument that a large fraction of the pentose 5-phosphate formed by the pentose pathway is recycled, i.e. the direction of carbon flux is from pentose to hexose in the non-oxidative segment of the pathway. The very high activity of the pentose cycle in liver and the slow rate of pentose 5-phosphate incorporation into nucleic acids indicates that nucleic acid synthesis is not the driving force which directs glucose carbon into the pentose cycle. The cycle in normal liver may be viewed as an "idiling" pathway which produces $CO_2$ and triose phosphate as by-products, and maintains the NADP/NADPH redox couple in the reduced state. The net formation of triose phosphate *via* the cycle is seen to be a means of by-passing the energy-requiring and regulatory enzyme phosphofructokinase in the glycolytic pathway. The triose phosphate may be directed into the lower half of the glycolytic pathway to produce ATP and into the tricarboxylic acid cycle for the same purpose or may be directed into glycerol or protein etc. It is likely, however, that under most conditions the regulation of the "idiling" of the pentose cycle in normal liver is through the NADP/NADPH redox couple and the associated de-inhibition mechanisms of glucose 6-phosphate dehydrogenase activity previously discussed (Section 1.2.1). Under conditions of increased demand for NADPH, for example when the rate of fatty acid synthesis is high due to a high dietary intake of carbohydrate, the "idiling" may be increased to accommodate the demand. The large increase in activity of

glucose 6-phosphate dehydrogenase under these dietary conditions supports this view. Thus an increased flux of glucose carbon through the pentose phosphate cycle in regenerating and neoplastic liver should occur only if there is a greater demand by these tissues for NADPH which cannot be met by other means such as 'malic' enzyme since the production of pentose 5-phosphate is far in excess of requirements.

## 8.5 The pentose phosphate pathway in regenerating liver

Following partial hepatectomy, the liver remnant is a rapidly growing tissue which regains its original mass in approximately 4 days (Bucher, 1963). It was considered that this rapid growth rate should result in a significant increase in the flux of carbon through the pentose phosphate pathway to meet the demands for NADPH required for reductive biosynthesis. This speculation is supported by the 2 fold increase in the maximum catalytic capacity of glucose 6-phosphate dehydrogenase, the regulatory enzyme of the oxidative segment of the pathway, in regenerating liver (Chapter 3). The results of $^{14}C$-isotope studies using [1-$^{14}C$] and [6-$^{14}C$] glucose (Chapter 4) may also be interpreted as implying an increased flux of glucose carbon through the pentose pathway. The C-1/C-6 ratio in $CO_2$ is 2.2 in regenerating liver compared to 1.9 in normal liver. As previously discussed, the C-1/C-6 ratio may represent various combinations of metabolic pathways since the ratio is a function of 3 independent variables. However, the similarity in absolute terms between the amounts of $^{14}C$-isotope recovered in $CO_2$ following the metabolism of

$^{14}$C-labelled glucose by the two cell types suggests that the metabolism is similar in these tissues. If it is assumed that the rate of glucose utilization, the specific radioactivity of glucose 6-phosphate and the extent of oxidation of C-3 of pyruvate is nearly the same in normal and regenerating liver, then the C-1/C-6 ratio may be interpreted as showing a slight increase in the metabolism of glucose 6-phosphate *via* the oxidative segment of the pentose pathway in regenerating liver. The C-6/C-1 ratio in lipid may also be seen from a comparative viewpoint as indicating that the flux of carbon through the pentose phosphate pathway is greater in regenerating liver than normal liver. The C-6/C-1 ratio is 2.9 for regenerating liver and 1.5 for normal liver. The increase in the ratio for regenerating liver may be taken to imply that more of the triose phosphate consisting of carbons 4, 5 and 6 of glucose formed by Reaction F of Fig. 1.3 is incorporated into lipid in regenerating liver than normal liver. This may be a consequence of increased flux of carbon through the oxidative segment of the pentose pathway and/or a decrease in the utilization of this triose phosphate for the resynthesis of hexose 6-phosphate *via* octulose 1,8 bisphosphate. This latter interpretation is consistent with the decrease in the pentose cycle activity treated in Chapter 7 and below. It is emphasized that the results discussed above and this interpretation apply only to the pentose pathway and not to the pentose cycle.

The results of the $^{14}$C-isotope redistributions described in Chapter 7 show that the F-type pentose cycle does not contribute significantly to glucose metabolism in

regenerating liver, that no net pentose phosphate formation occurs non-oxidatively, and that the percentage of glucose metabolized *via* the L-type pentose cycle in this tissue is approximately 11%. Thus, assuming glucose utilization to be the same in normal and regenerating liver, the amount of glucose 6-phosphate metabolized through the L-type pentose cycle is 1/2 to 1/3 that of normal liver. Even if glucose utilization is not the same in regenerating and normal liver, there is no selective activation of the pentose phosphate cycle in regenerating liver. This observation is not necessarily in conflict with the conclusions reached above regarding the flux of carbon through the pentose phosphate pathway. The $^{14}C$-isotope distribution data (Chapter 7) result in estimates of the complete L-type pentose cycle, i.e. from glu 6-P to pentose 5-P to glu 6-P, whereas the $^{14}CO_2$ and $^{14}C$-lipid data reflect only segments of the cycle, i.e. from glu 6-P to pentose 5-P and $CO_2$ and from glu 6-P to pentose 5-P and $CO_2$ to triose-P. One obvious interpretation of these results is that a smaller fraction of the glucose carbon entering the oxidative segment of the pentose pathway completes the cycle in regenerating liver relative to normal liver.

The decrease in total pentose cycle flux (but not pathway flux) may be the result of either a block in the flow of carbon at a regulatory site in the non-oxidative segment of the cycle and/or an increased utilization of one or more of the intermediates of the pathway *via* a sequence of reactions other than those which result in hexose 6-phosphate formation. If a block in the flux of carbon occurred at a regulatory enzyme, the result would be an

accumulation of the intermediates preceding this reaction in the pathway. The intermediate concentrations of 24h regenerating liver shown in Chapter 3 indicate an increase in the concentration of heptulose 7-phosphate (relative to normal liver). This could be taken to indicate that the phosphotransferase enzyme acts as a rate-limiting regulatory enzyme in the non-oxidative segment of the pathway. Unfortunately, the concentrations of the other substrate, octulose 1,8 bisphosphate and of the products of the reaction, heptulose 1,7 bisphosphate and octulose 8-phosphate, are not available and no definite conclusions regarding the regulation of the pathway at this point can be made. The concentrations of all three pentose 5-phosphates are much reduced in regenerating liver relative to normal liver. This observation argues against blockage of flux at the phosphotransferase catalyzed reaction unless it is assumed that a large proportion of pentose 5-phosphate accumulates as octulose 1,8 bisphosphate. It is also of note that the capacity of enzyme preparations from regenerating liver to catabolize ribose 5-phosphate and the rate of hexose 6-phosphate formation *in vitro* is not significantly different from normal liver. Thus it is considered that a block of carbon flux in the non-oxidative segment of the pathway is unlikely to be responsible for the marked decrease in pentose cycle flux. In the absence of a block in flux through the reactions of the pathway, a number of alternative possibilities could explain the failure to observe complete pentose cycle activity in regenerating liver consistent with the flux of carbon through the oxidative segment of the cycle. Any such mechanism

need only show that formation of glucose 6-phosphate from pentose 5-phosphate is reduced in regenerating liver. Thus the failure to achieve isotopic equilibrium between fructose 6-phosphate and glucose 6-phosphate *via* the phosphoglucose isomerase catalyzed reaction could account for the observation that less $^{14}C$-isotope is incorporated into C-2 of glucose 6-phosphate from [5-$^{14}C$] glucose. Phosphoglucose isomerase is known to be inhibited by both 6-phosphogluconate and erythrose 4-phosphate and therefore this enzyme is a possible site of regulation in terms of pentose cycle activity. Alternately, the utilization of fructose 6-phosphate or any other of the intermediates of the pathway *via* other metabolic routes could act to interrupt the flow of carbon through the pentose cycle. The incorporation of $^{14}C$-isotope into acid insoluble cell components (Chapter 4) indicates that there is an increase in glucose carbon flux into nucleic acids in regenerating liver. This observation implies that isolated cells from regenerating liver incubated under the conditions used for these experiments reflect in part the potential of these cells for growth. However, as discussed above, the amount of carbon required for nucleic acid synthesis is small relative to pentose pathway flux and cannot of itself account for the decreased pentose cycle activity in regenerating liver. The incorporation of $^{14}C$-isotope into protein and lipid are not increased in regenerating liver compared to normal and therefore give no indication of the direction of flow of glucose carbon out of the non-oxidative segment of the pentose pathway, i.e. if the carbon is not recycled to hexose 6-phosphate from pentose

5-phosphate and is being directed into other metabolic pathways, no information regarding the end products of this redirection can be gained from the data of Chapter 4.

In summary the results as interpreted here indicate that the pentose phosphate pathway flux in regenerating liver is nearly the same or moderately increased relative to normal liver but that the pentose cycle activity is decreased to 1/2 or 1/3 that of normal liver. The break in the pentose cycle could occur at one or more of the reactions of the non-oxidative segment of the pathway following the formation of heptulose 7-phosphate and triose-phosphate by the transketolase reaction between xylulose 5-phosphate and ribose 5-phosphate. It is not possible based on the data available to determine at what point or points in the reaction sequence of the non-oxidative segment of the pentose pathway the flow of pentose 5-phosphate to hexose 6-phosphate is interrupted or where the glucose carbon is directed. The important conclusion with respect to the questions posed in this thesis is that it does not appear to be necessary to increase the flow of glucose carbon through the pentose phosphate pathway to any significant extent to accommodate the growth and replication of regenerating liver cells.

## 8.6 The pentose phosphate cycle in hepatomas

The activity of the pentose phosphate pathway or cycle in hepatomas relative to normal liver is difficult to assess based on the results discussed in this thesis. The alterations in the enzyme profile indicate an increased capacity for flux of carbon through the oxidative segment

of the pathway in both Morris transplantable hepatomas studied. The activity of the non-oxidative segment of the cycle as measured by the rate of ribose 5-phosphate catabolism and hexose 6-phosphate formation, however, is moderately greater in hepatoma 7777 and slightly decreased in hepatoma 5123C (Section 3.6). The incorporation of $^{14}C$-isotope into $CO_2$ from [1-$^{14}C$] and [6-$^{14}C$] glucose also implies an increased oxidative segment flux in hepatoma 5123TC, however the C-1/C-6 ratio must be interpreted with some caution particularly in light of the very low levels of $^{14}CO_2$ produced from [6-$^{14}C$] glucose. The $^{14}CO_2$ data indicates that the activity of the tricarboxylic acid cycle is very low in the hepatoma cells under these incubation conditions and the high C-1/C-6 ratio in $^{14}CO_2$ only indicates that the decarboxylation of C-1 of glucose 6-phosphate *via* the oxidative segment of the pentose pathway is much greater (approx. 10 fold) than decarboxylation of C-3 of pyruvate *via* the tricarboxylic acid cycle and does not reflect the activity of the pentose cycle relative to total glucose metabolism. The low activity of the tricarboxylic acid cycle implies that the hepatoma cells derive most of their ATP *via* the glycolytic pathway. The C-6/C-1 ratio in lipid of 0.95 is consistent with the view that the triose phosphate pool is derived largely from the glycolytic pathway and therefore will be equally labelled from both [1-$^{14}C$] and [6-$^{14}C$] glucose. Since an estimate of net glucose utilization can be made with hepatoma cells, it is of interest to compare the rate of $^{14}CO_2$ production to the rate of glucose utilization. When [1-$^{14}C$] glucose is substrate the rate of $^{14}CO_2$ production was 2.08 µg atom/g/h

and the rate of glucose utilization 30 μmoles/g/h. Assuming all $^{14}CO_2$ was formed by the oxidative segment of the pentose phosphate pathway, then only 6.7% of the utilized glucose enters the oxidative segment of the pathway. If the dilution of the hexose 6-phosphate pool due to recycling is not considered, then roughly 2% of the utilized glucose is metabolized to triose phosphate and $CO_2$ *via* the pentose cycle by this hepatoma. Thus the contribution of the oxidative segment of pentose pathway to glucose metabolism is considered to be small relative to total glucose metabolism. Unfortunately it is not possible to compare actual rates of flux of glucose metabolism *via* the oxidative segment of the pathway in the hepatoma with rates found in normal liver as a consequence of the difficulties of measuring glucose "utilization" by normal liver cells (see Section 4.9).

The analysis of the $^{14}C$-isotope distribution in the carbon atoms of glucose 6-phosphate following the metabolism of [2-$^{14}C$] and [5-$^{14}C$] glucose does little to resolve the question of the activity of the pentose cycle in hepatoma cells. The distribution following [5-$^{14}C$] glucose metabolism is similar to that of normal liver and results in an estimate of 42.9% L-type pentose cycle. However, the distribution of $^{14}C$-isotope following the metabolism of [2-$^{14}C$] glucose indicates that the L-type pentose cycle is not the only and may not be the most important series of reactions redistributing $^{14}C$-isotope and these other $^{14}C$-isotope randomizing reactions will act to modify the estimate of the L-type pentose cycle. The results discussed in Section 7.6 indicate that numerous reactions which result

in the transfer of $^{14}C$-isotope from C-2 of glucose are very active in hepatoma cells. It is not possible to make an accurate estimate of the activity of the L-type pentose cycle in the presence of this large background of $^{14}C$-isotope randomization. Therefore, the best estimate of the activity of the pentose phosphate pathway in these cells must be that based on the $^{14}CO_2$ data which indicate only a small contribution of the pathway relative to total glucose metabolism. The proposal of Weber *et al.* (1973) that the flux of glucose carbon into pentose phosphate is increased by both the oxidative and non-oxidative pathways is not supported by these results. The extensive redistribution of isotope in the carbon atoms of glucose 6-phosphate (Chapter 7) indicates that a large proportion of the pentose 5-phosphate is recycled to hexose 6-phosphate which implies that the direction of flux is from pentose to hexose in the non-oxidative segment of the pathway. Although there is no way of determining whether there is any increased net flux of glucose carbon into pentose *via* the oxidative segment of the cycle, since we do not have a normal liver value for comparison, it is clear that the pentose phosphate pathway is not selectively activated in these cells, rather it appears that the major alteration of glucose metabolism in the hepatoma is concerned with the energy producing pathways, i.e. the glycolytic pathway and the tricarboxylic acid cycle.

## 8.7 Retrodifferentiation, foetal liver and the pentose phosphate pathway

The existence of undifferentiated forms of enzymes

in foetal, regenerating and neoplastic liver tissues provoked the speculation early in this work that perhaps the F-type pentose pathway mechanism represented a foetal form of the pathway and that retrodifferentiation might result in a re-expression of this pathway in regenerating and/or neoplastic tissues. The results of these studies do not support this proposal. The similarity between normal, foetal and regenerating liver and the hepatomas in terms of the carbon deficit following ribose 5-phosphate metabolism (Chapter 3) and the capacity to utilize D-*glycero*-D-*ido*-octulose 1,8 bisphosphate for hexose 6-phosphate synthesis indicate the potential operation of the L-type pathway in all hepatic tissues. The case for the operation of the L-type reaction sequence in regenerating liver and hepatoma 5123TC is strongly supported by the $^{14}C$-isotope distribution following the metabolism of [5-$^{14}C$] glucose (Chapter 7). On the basis of this data it is tentatively concluded that the L-type pentose cycle mechanism operates in all tissues studied.

The quantitative contribution of the pentose cycle in foetal liver cannot be accurately estimated based on the data presented here. However, the similarity between the enzyme activity profile (Chapter 3) and the $^{14}C$-isotope distribution data of Crockett and Leslie (1963, see Chapter 4) with that of regenerating liver suggest a similar pattern of glucose metabolism in these two tissues.

## 8.8 Concluding statement

The analysis of changes in flux of a particular metabolic pathway in tissues in the presence of alternative

reaction sequences utilizing the same intermediates is a complex and difficult task. The argument that the physiological role of the pentose phosphate pathway is the provision of metabolic intermediates and that these intermediates are used primarily for biosynthesis implies a relationship between the two processes but this is difficult to prove experimentally. The studies described in this thesis have utilized many of the experimental approaches which are the stock in trade of intermediary metabolism, however the results from each investigation taken individually are subject to numerous interpretations. It is the curse of the biochemist in intermediary metabolism that experiments can rarely be designed which can yield unequivocal answers particularly to questions relating to metabolic pathway fluxes. Rather, it is usually the case that a series of experiments yielding individual equivocal results but indicating a trend when taken collectively are the basis for our understanding of intermediary metabolism. The conclusions reached as a result of the studies described here must fall into that category. The interpretation of $^{14}C$-isotope data in Chapter 4 highlights the limitations of the use of isotopes for studying metabolism *via* the pentose phosphate pathway. The experimental approaches used in the remainder of the thesis and based on the theoretical analysis detailed in Chapter 5 allow for a more meaningful interpretation of $^{14}C$-isotope data, however it is recognized that this approach too has severe limitations. This is best illustrated by the inability to interpret the quantitative participation of the L-type pentose cycle in hepatoma cells.

The central purpose of this work has been to determine if there is any relationship between cell growth and proliferation and the flux of carbon through the pentose phosphate pathway. The results indicate that there is no significant correlation between the two processes. The requirement for pentose 5-phosphate for nucleotide and nucleic acid synthesis can be adequately supplied by the pathway flux in normal non-growing tissue. The provision of reducing equivalents as NADPH was considered to be the most likely driving force behind a redirection of glucose 6-phosphate into the pentose phosphate pathway. However, the requirement of growing tissues for NADPH is largely a generalization and cannot be quantitatively assessed. Further, the supply of NADPH *via* the pentose phosphate pathway may not be the important regulator in growth situations, rather the distribution of NADPH to specific reductive biosynthetic processes required for cell growth and division and away from non-essential reactions may act to regulate NADPH levels as required. Alternately NADPH production by other mechanisms such as "malic" enzyme or cytoplasmic transhydrogenase may play an important role in the provision of reducing equivalents. It is clear that the flux of glucose carbon through the oxidative segment of the pentose phosphate pathway is regulated to a large extent by the NADP/NADPH redox couple, however it appears that rapid rates of cellular proliferation do not result in a selective activation of the pathway in either normal or abnormal growth situations.

# APPENDIX

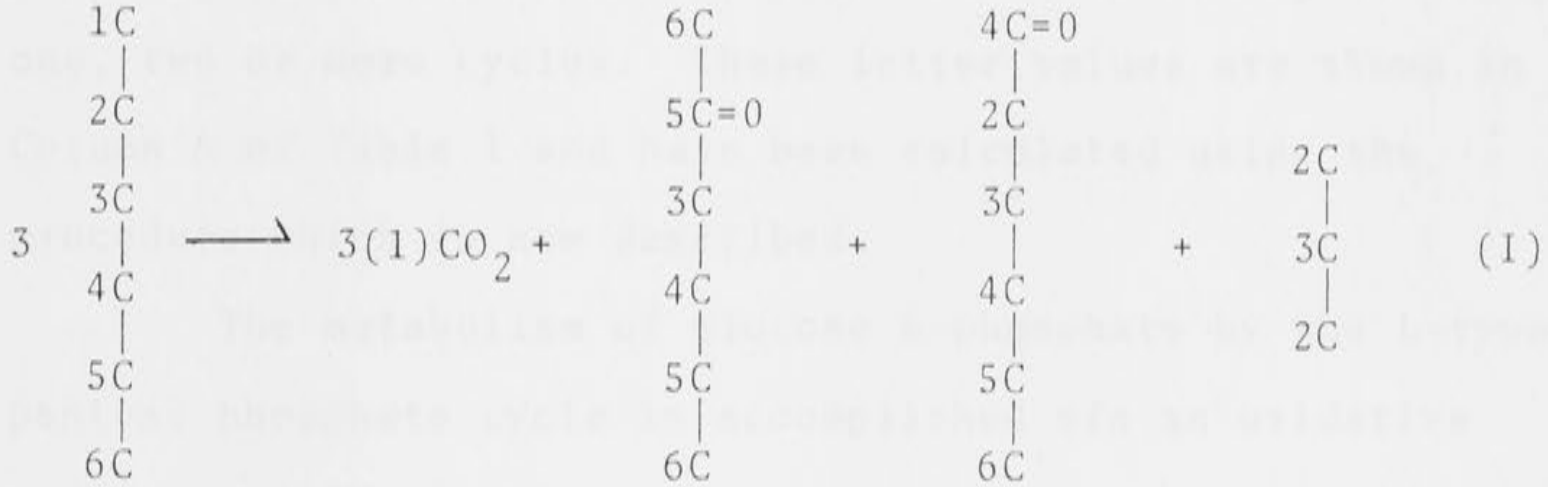
A method for estimating the quantitative participation of the L-type pentose cycle is described. The assumptions and limitations of this approach are the same as those discussed in Chapter 5. The following presents tabulations of the predicted specific radioactivities of the carbon atoms of glucose 6-phosphate following the metabolism of [5-$^{14}$C] and [4,5,6-$^{14}$C] glucose. The latter analysis illustrates the predicted $^{14}$C incorporation into carbon 1 of glucose 6-phosphate from C-4 and C-6 of glucose as discussed in Section 7.7.

## The distribution of $^{14}$C from [5-$^{14}$C] glucose by reactions of the L-type pentose phosphate cycle

The redistribution of the carbon atoms of glucose by the reactions of the non-oxidative segment of the L-type pentose phosphate cycle is shown in Fig. 5.1. It is significant that formation of hexose 6-phosphate by these reactions leads to the ordered redistribution of carbon atoms 4,5 and 6 of the original glucose molecule into the top three carbons of hexose 6-phosphate. This distribution is unique and distinguishes between the L-type and F-type mechanisms for the non-oxidative segment of the cycle. The estimate is based on the use of [5-$^{14}$C] glucose as substrate and the unique relocation of $^{14}$C from position 5 into position 2 of glucose 6-phosphate (Fig. 5.1). The metabolism of [5-$^{14}$C] glucose by the test tissue is then followed by the isolation and degradation of the glucose moiety of glucose 6-phosphate (Williams *et al.*, 1978a).

The distribution of $^{14}C$ in C-2 of glucose 6-phosphate thus serves as a significant test of the operation of the L-type pentose cycle and as the measure of its relative contribution to total glucose metabolism.

The case in which glucose is metabolized wholly by the L-type pentose cycle will be considered first. In the following analysis it will be assumed that isotopic equilibrium exists between glucose 6-phosphate and fructose 6-phosphate and also between dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. The net conversion to be considered is shown in Reaction I (see Fig. 5.1).




3 Glc 6-P → $3CO_2$ + Fru 6-P + Glc 6-P + Grn-P

The redistribution of carbon 5 into position 2 of fructose 6-phosphate originates from the aldolase condensation (Reaction H, Fig. 5.1) of arabinose 5-phosphate with the dihydroxyacetone phosphate which is formed in Reactions F and G catalyzed by transketolase and triose phosphate isomerase respectively. If the specific radioactivity of carbon 5 of glucose is defined as 100, then the specific radioactivity of position 2 of hexose 6-phosphate formed at the end of one turn of the cycle is 50. If the glucose 6-phosphate is passed through the cycle a second time, the specific radioactivity of position 2 becomes 75. The

changes in specific radioactivity of position 2 of hexose 6-phosphate which will occur during the third, fourth, fifth and subsequent cycles may be calculated and are shown under Column B of Table 1.

It is theoretically possible to determine the average distribution of $^{14}C$ in the carbon atoms of hexose 6-phosphate at steady state from the following values listed in Table 1. The first value expresses the concentration of $^{14}C$ in the various carbon atoms at each turn of the cycle and is shown in Column B of Table 1. The second value represents the amount of each $^{14}C$-labelled species of hexose 6-phosphate in the pool which has undergone zero, one, two or more cycles. These latter values are shown in Column A of Table 1 and have been calculated using the procedure which is now described.

The metabolism of glucose 6-phosphate by the L-type pentose phosphate cycle is accomplished *via* an oxidative and a non-oxidative series of reactions as shown in Reactions II and III.

Oxidative reactions

$$3 \text{ Glucose 6-P} + 6 \text{ NADP}^+ \longrightarrow 3CO_2 + 3 \text{ Pentose 5-P} + 6 \text{ NADPH} + 6H^+ \quad \text{(II)}$$

Non-oxidative reactions

$$3 \text{ Pentose 5-P} \longrightarrow \text{glucose 6-P} + \text{fructose 6-P} + \text{dihydroxyacetone-P} \quad \text{(III)}$$

Equilibration by phosphoglucoseisomerase

$$\text{Glucose 6-P} + \text{fructose 6-P} \rightleftharpoons 2 \text{ glucose 6-P} \quad \text{(IV)}$$

Following the equilibration of the glucose 6-phosphate and fructose 6-phosphate formed (Reaction IV), the sum reaction for the cycle is shown in Reaction I.

Table 1: The redistribution of $^{14}C$ from [5-$^{14}C$] glucose into glucose 6-phosphate when all glucose is metabolized by the L-type pentose cycle

| Number of cycles | Fraction of hexose 6-phosphate in indicated cycle | Concentration of $^{14}C$ in hexose 6-phosphate of indicated cycle | | Relative specific radioactivity of C-2 and C-5 of hexose 6-phosphate | |
|---|---|---|---|---|---|
| | A | B | | C (A x B) | |
| | | C-2 | C-5 | C-2 | C-5 |
| 0 | 0.333 | 0 | 100 | 0 | 33.333 |
| 1 | 2/3 x 1/3 = 0.2222 | 50.000 | 100 | 11.110 | 22.222 |
| 2 | $(2/3)^2$ x 1/3 = 0.1482 | 75.000 | 100 | 11.115 | 14.820 |
| 3 | $(2/3)^3$ x 1/3 = 0.0988 | 87.500 | 100 | 8.645 | 9.880 |
| 4 | $(2/3)^4$ x 1/3 = 0.0658 | 93.750 | 100 | 6.168 | 6.580 |
| 5 | $(2/3)^5$ x 1/3 = 0.0439 | 96.870 | 100 | 4.253 | 4.390 |
| 6 | $(2/3)^6$ x 1/3 = 0.0293 | 98.430 | 100 | 2.884 | 2.930 |
| 7 | $(2/3)^7$ x 1/3 = 0.0198 | 99.210 | 100 | 1.964 | 1.980 |
| 8 | $(2/3)^8$ x 1/3 = 0.0129 | 99.600 | 100 | 1.285 | 1.290 |
| 9 | $(2/3)^9$ x 1/3 = 0.0086 | 99.790 | 100 | 0.858 | 0.860 |
| 10 | $(2/3)^{10}$ x 1/3 = 0.0058 | 99.890 | 100 | 0.577 | 0.578 |
| | | Steady state total relative specific activity = 49.999 | | | 100.005 |

Calculations show the distributions of $^{14}C$-isotope into carbon 2 and carbon 5 of hexose 6-phosphate at steady state, when [5-$^{14}C$] glucose is the substrate and glucose metabolism occurs solely by the pentose cycle. The relative specific radioactivity of carbon 5 of glucose is defined as 1.

Thus, like the F-type cycle, 3 molecules of glucose 6-phosphate are required to enter the cycle for every molecule of glucose 6-phosphate metabolized to $CO_2$ and triose phosphate. This stoichieometry indicates that at steady state 1/3 of the hexose 6-phosphate of the pool would continually be converted to triose phosphate and $CO_2$ and would be replenished by the phosphorylation of glucose. Therefore 1/3 of the hexose 6-phosphate pool will have undergone zero cycles and 2/3 will have undergone one or more cycles. Thus 1/3 of the pool is shown in Column A of Table 1 for zero cycle. When this amount passes through the cycle, 2/3 of it will remain as hexose 6-phosphate, the remainder will have been converted to triose phosphate and $CO_2$. Thus 1/3 of 2/3 is the fraction which has undergone 1 cycle. Similarly, the fraction which has undergone two, three, four or more cycles may be calculated.

The contribution of $^{14}C$ from each species of hexose 6-phosphate in the pool is shown in Column C of Table 1 when [5-$^{14}C$] glucose is metabolized. The values in Column C were obtained by multiplying the concentration of $^{14}C$ in each species (Column B) by the amount of that species in the pool (Column A). The sum of these values gives the average concentration of $^{14}C$ in carbon atoms 2 and 5 of hexose 6-phosphate of the pool at steady state when all glucose is metabolized entirely by the L-type pentose cycle. The ratio of the relative specific radioactivities of carbon 2 to carbon 5 is shown to be 0.50 when all glucose is metabolized *via* the L-type pentose cycle.

The distribution of $^{14}C$ in hexose 6-phosphate when metabolism occurs by the L-type pentose cycle and other dissimilating pathways

It has been assumed in the above treatment that the inflowing glucose is specifically labelled in carbon 5, that it has a specific radioactivity of 100, and that the influx of glucose is exactly equal to the outflow of glucose 6-phosphate by way of oxidation to $CO_2$ and triose phosphate by the pentose cycle of Fig. 5.1 and Reaction I. Under conditions where glucose is converted to glycogen etc. and/or when glycolysis contributes to metabolism, then the outflow and replacement of hexose 6-phosphate is greater and the degree of $^{14}C$ distribution is modified. The effects of glucose metabolism by these alternate pathways on the estimation of the contribution of the L-type pentose cycle are now considered.

When the utilization of glucose proceeds 20% by the L-type pentose cycle and 80% by other pathways, the fraction of total hexose 6-phosphate in the pool which has undergone 1 or more cycles is 0.286. This is so because a 20% utilization of glucose by the cycle requires 60 molecules of glucose to be metabolized in the cycle yielding 60 molecules of $CO_2$, 20 molecules of dihydroxyacetone phosphate and 40 molecules of hexose 6-phosphate (Fig. A1). At steady state 100 parts of hexose 6-phosphate will arise from [5-$^{14}C$] glucose and will be labelled with $^{14}C$ exclusively in carbon 5, while 40 parts will be regenerated *via* the cycle and $^{14}C$-isotope will be redistributed into carbon 2. The fraction of the pool which has undergone zero cycles is 100/140 or 0.714 and the fraction which has

Fig A 1

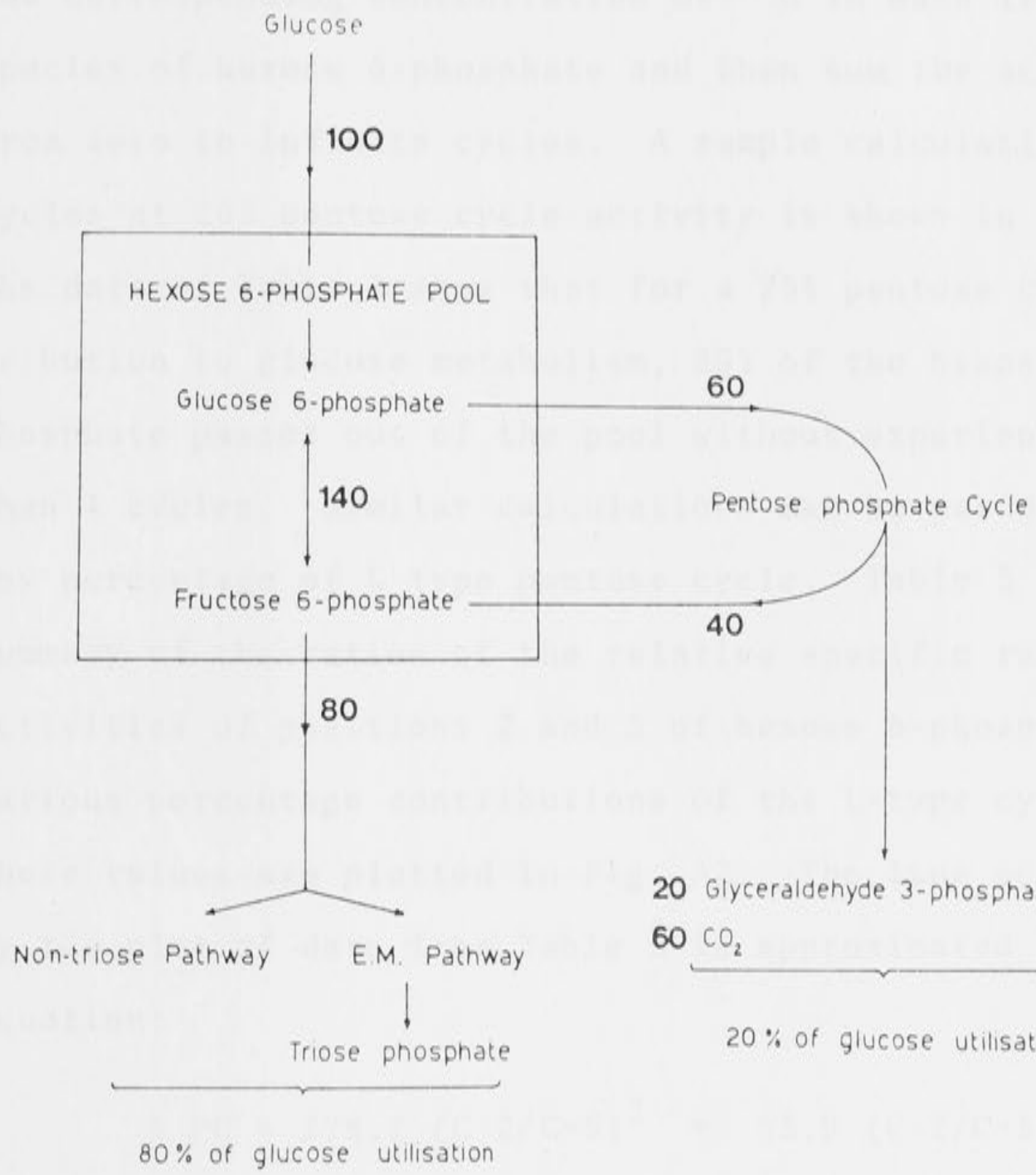



Fig. A.1: Glucose 6-phosphate metabolism when 20% occurs *via* the pentose phosphate cycle

The flow of hexose 6-phosphate carbon from the hexose 6-phosphate pool when metabolism is 20% *via* the pentose phosphate cycle and 80% *via* other pathways is shown. The net flux of glucose metabolism is assumed to be 100. Under these conditions the steady state hexose 6-phosphate pool size is 140.

gone through one or more cycles is 40/140 or 0/286 (see above). Of the cycled fraction, 0.286 of 0.714 has been cycled twice etc. In order to calculate the total contribution of carbon 5 of glucose to positions 5 and 2 of hexose 6-phosphate, it is necessary to multiply the fraction of the total pool contributed by each cycle with the corresponding concentration of $^{14}C$ in each labelled species of hexose 6-phosphate and then sum the activity from zero to infinite cycles. A sample calculation for 7 cycles at 20% pentose cycle activity is shown in Table 2. The data of Table 2 show that for a 20% pentose cycle contribution to glucose metabolism, 99% of the hexose 6-phosphate passes out of the pool without experiencing more than 4 cycles. Similar calculations may be performed for any percentage of L-type pentose cycle. Table 3 shows a summary of the ratios of the relative specific radioactivities of positions 2 and 5 of hexose 6-phosphate for various percentage contributions of the L-type cycle. These values are plotted in Fig. A2. The line generated by the plot of data from Table 3 is approximated by the equation:

$$\% PC = 278.2 (C\text{-}2/C\text{-}5)^2 + 55.9 (C\text{-}2/C\text{-}5) + 1.4$$

The error of the equation is approximately 1.5% and is contributed when values of less than 20% L-type pentose cycle are measured. The error decreases to zero for pentose cycle values greater than 20%.

Table 2: The redistribution of $^{14}C$ from [5-$^{14}C$] glucose into glucose 6-phosphate when 20% of glucose metabolism occurs by the L-type pentose cycle

| Number of cycles | Fraction of hexose 6-phosphate in indicated cycle | Concentration of $^{14}C$ in hexose 6-phosphate of indicated cycle | | Relative specific radioactivity of C-2 and C-5 of hexose 6-phosphate | |
|---|---|---|---|---|---|
| | A | B | | C (A x B) | |
| | | C-2 | C-5 | C-2 | C-5 |
| 0 | $(0.286)^0 \times 0.714 = 0.7140$ | 0 | 100 | 0 | 71.400 |
| 1 | $(0.286)^1 \times 0.714 = 0.2040$ | 50 | 100 | 10.200 | 20.400 |
| 2 | $(0.286)^2 \times 0.714 = 0.0583$ | 75 | 100 | 4.372 | 5.830 |
| 3 | $(0.286)^3 \times 0.714 = 0.0167$ | 87.5 | 100 | 1.461 | 1.670 |
| 4 | $(0.286)^4 \times 0.714 = 0.0048$ | 93.75 | 100 | 0.450 | 0.480 |
| 5 | $(0.286)^5 \times 0.714 = 0.0013$ | 96.87 | 100 | 0.126 | 0.130 |
| 6 | $(0.286)^6 \times 0.714 = 0.0004$ | 98.43 | 100 | 0.040 | 0.040 |
| 7 | $(0.286)^7 \times 0.714 = 0.0001$ | 99.21 | 100 | 0.010 | 0.010 |
| | | Steady state total relative activity | | 16.659 | 99.960 |

Calculations show the distributions of $^{14}C$-isotope into carbon 2 and carbon 5 of hexose 6-phosphate at steady state when [5-$^{14}C$] glucose is the substrate and 20% of glucose is metabolized by the pentose cycle and 80% by all other pathways. The relative specific radioactivity of carbon 5 of [5-$^{14}C$] glucose is taken to be 100.

Table 3: Percentage L-type pentose cycle as a function of the specific ratios of radioactivity in glucose 6-phosphate following the metabolism of [5-$^{14}$C] glucose

| Percentage pentose cycle | Ratio of C-2/C-5 in hexose 6-phosphate from [5-$^{14}$C] glucose |
|---|---|
| 0 | 0 |
| 10 | 0.091 |
| 20 | 0.167 |
| 30 | 0.231 |
| 40 | 0.289 |
| 50 | 0.332 |
| 60 | 0.374 |
| 70 | 0.410 |
| 80 | 0.443 |
| 90 | 0.469 |
| 100 | 0.500 |

The table shows the various percentage values for the L-type pentose cycle and their relation to the C-2/C-5 ratios of $^{14}$C radioactivity in glucose 6-phosphate following the metabolism by the test tissue of [5-$^{14}$C] glucose.

Fig. A.2: The percentage contribution of the L-type pentose cycle expressed as a function of C-2/C-5 ratios in glucose 6-phosphate

The plot shows the percentage of total glucose metabolism *via* the pentose cycle as a function of the theoretical values for the ratio of the relative specific radioactivities of carbon 2 to carbon 5 of hexose 6-phosphate when [5-$^{14}$C] glucose is metabolized by the L-type pentose cycle.

The curve is approximated by an equation of the form $y = Ax^2 + Bx + C$ (see text for discussion).

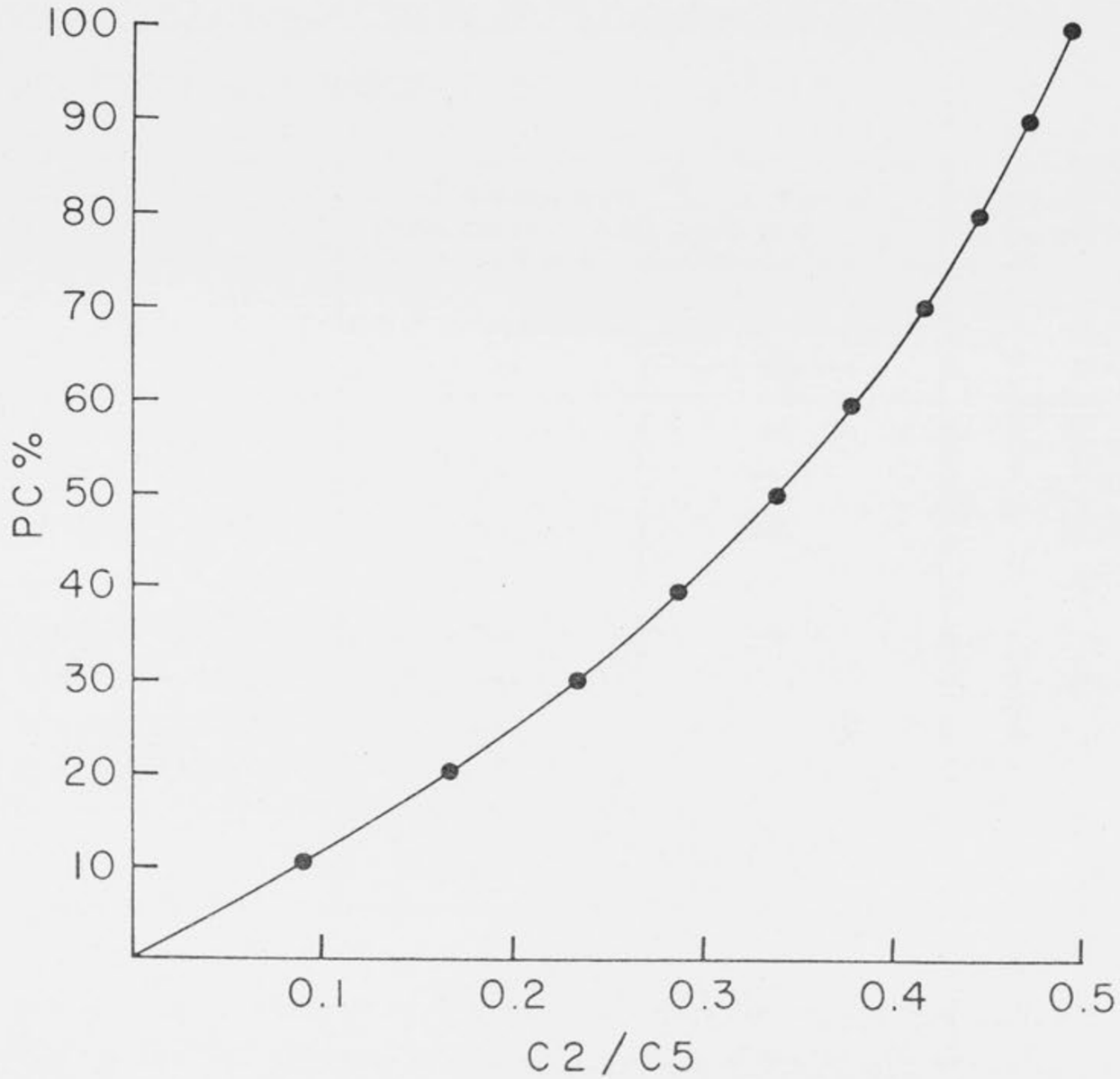



Fig A 2

Table 4 shows the contribution of C-6 and C-4 of glucose to position 1 of glucose 6-phosphate when 20% of glucose is metabolized by the L-type pentose cycle. The table shows that the C-1/C-6 or C-1/C-4 ratio is 0.14, compared to a C-2/C-5 ratio of 0.17 (Table 2). Thus, when [4,5,6-$^{14}$C] glucose is substrate, the ratio of specific radioactivity in C-1 to C-2 should be: 0.14 x 2/0.17 = 1.65 assuming the initial specific radioactivity of carbons 4,5 and 6 to be equal. As discussed in Section 7.7 this predicted accumulation of isotope is not observed.

Table 4: The redistribution of $^{14}C$ from [4-$^{14}C$] or [6-$^{14}C$] glucose into glucose 6-phosphate when 20% of glucose metabolism occurs by the L-type pentose cycle

| Number of cycles | Fraction of hexose 6-phosphate in indicated cycle | Concentration of $^{14}C$ in hexose 6-phosphate of indicated cycle | | Relative specific radioactivity of C-2 and C-5 of hexose 6-phosphate | |
|---|---|---|---|---|---|
| | A | B | | C (A x B) | |
| | | C-1 | C-6 or C-4 | C-2 | C-5 |
| 0 | $(0.286)^0 \times 0.714 = 0.7140$ | 0 | 100 | 0 | 71.400 |
| 1 | $(0.286)^1 \times 0.714 = 0.2040$ | 50 | 100 | 10.200 | 20.400 |
| 2 | $(0.286)^2 \times 0.714 = 0.0583$ | 50 | 100 | 2.915 | 5.830 |
| 3 | $(0.286)^3 \times 0.714 = 0.0167$ | 50 | 100 | 0.835 | 1.670 |
| 4 | $(0.286)^4 \times 0.714 = 0.0048$ | 50 | 100 | 0.240 | 0.480 |
| 5 | $(0.286)^5 \times 0.714 = 0.0013$ | 50 | 100 | 0.065 | 0.130 |
| 6 | $(0.286)^6 \times 0.714 = 0.0004$ | 50 | 100 | 0.020 | 0.040 |
| 7 | $(0.286)^7 \times 0.714 = 0.0001$ | 50 | 100 | 0.005 | 0.010 |
| | | Steady state total relative activity | | 14.280 | 99.960 |

Calculations show the distributions of $^{14}C$-isotope into carbon 1 and carbon 6 or 4 of hexose 6-phosphate at steady state when [6-$^{14}C$] or [4-$^{14}C$] glucose is the substrate and 20% of glucose is metabolized by the pentose cycle and 80% by all other pathways. The relative specific radioactivity of carbon 6 or 4 of [6-$^{14}C$] or [4-$^{14}C$] glucose is taken to be 100.

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